

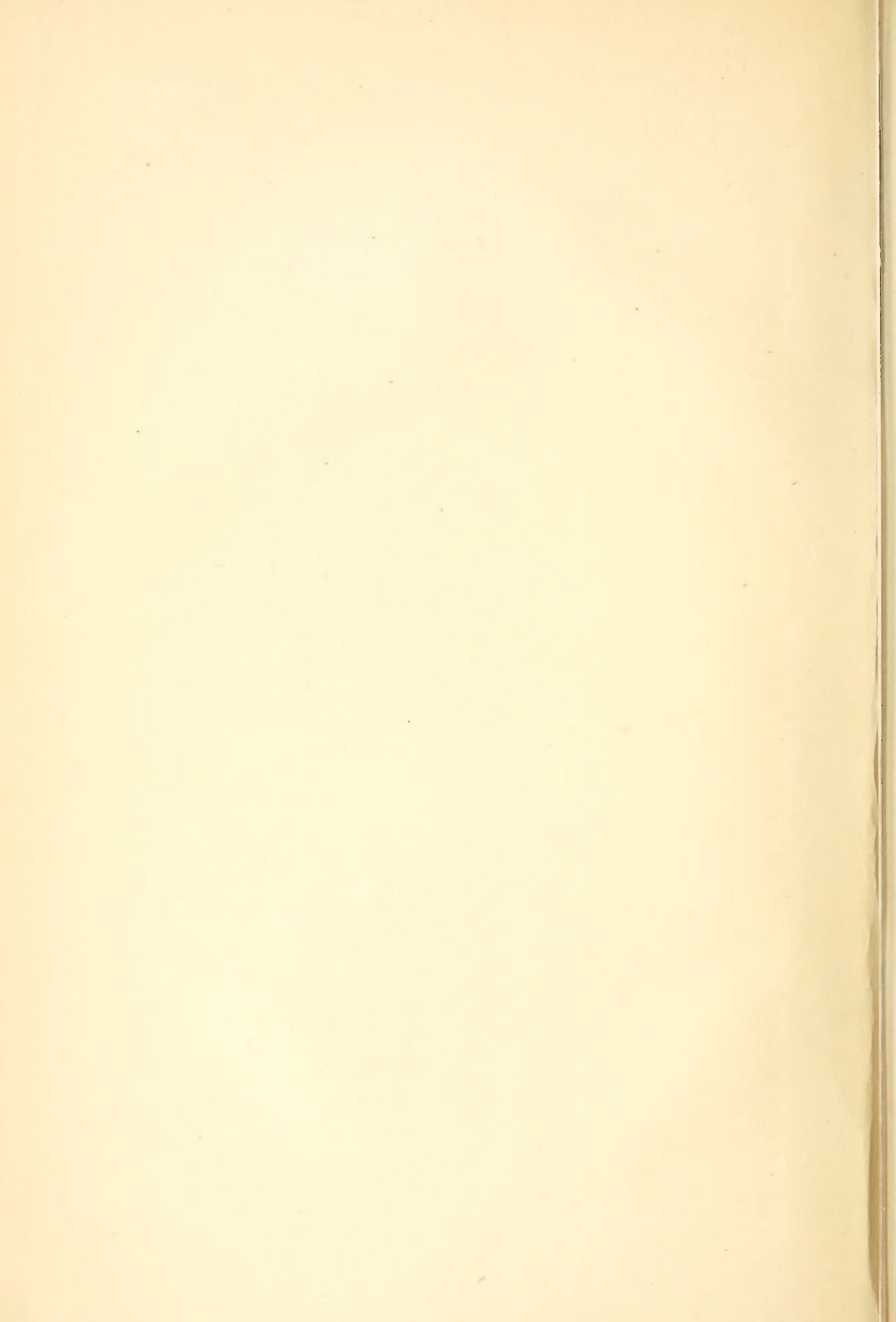


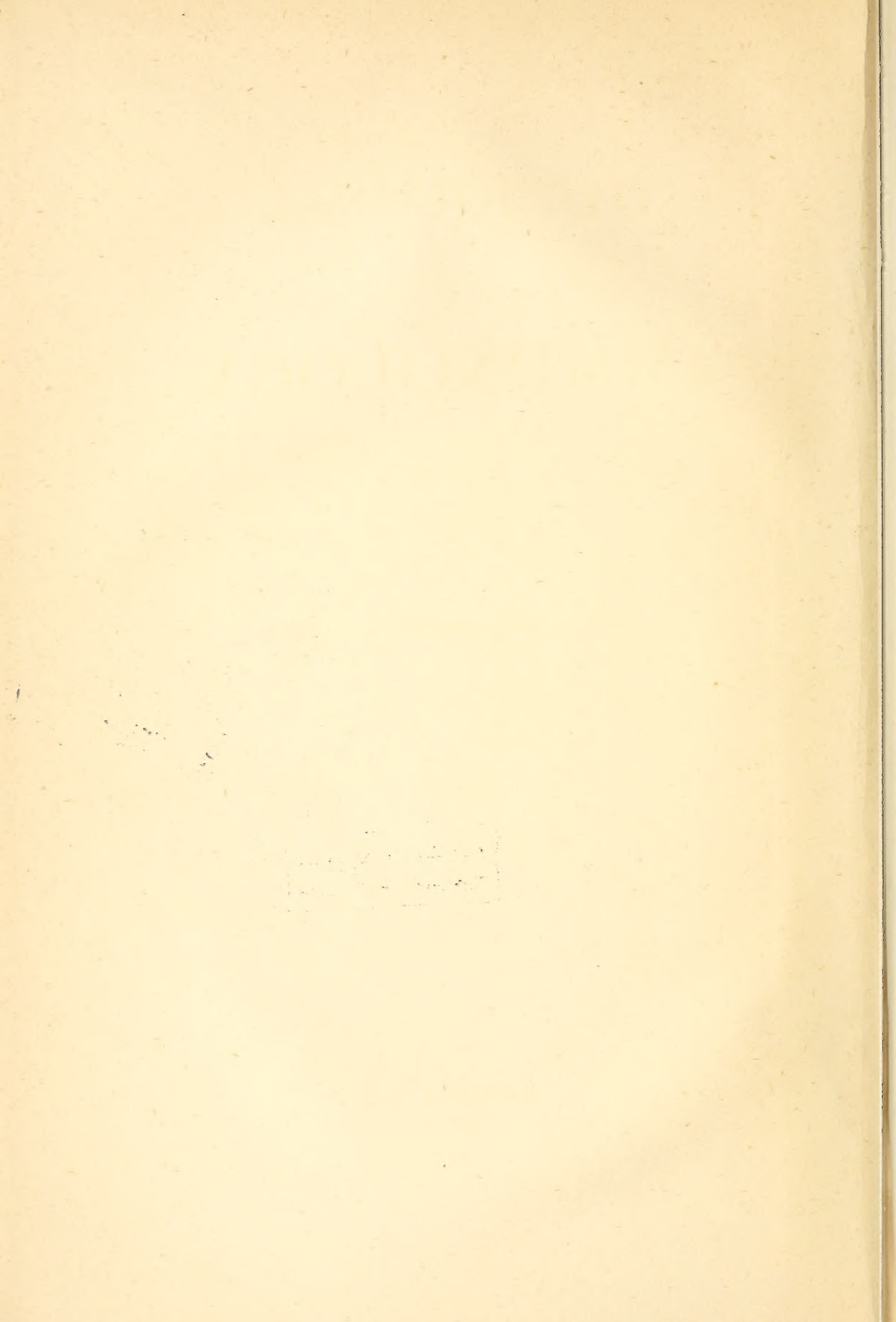
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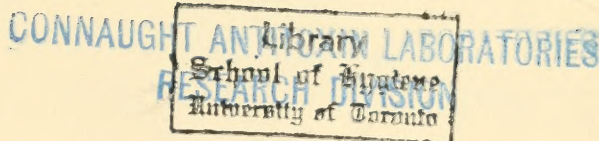




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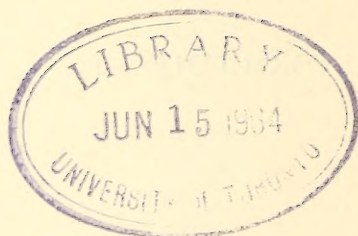
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MICROBAL STUDIES ON ACUTE RESPIRATORY INFECTION WITH ESPECIAL CONSIDERATION OF IMMUNOLOGICAL TYPES¹

WILLIAM H. PARK, ANNA W. WILLIAMS AND CHARLES KRUMWIEDE

Received for publication December 14, 1920

INTRODUCTION

Outline of work planned, discussion of results and general conclusion

It was decided at a conference of the members of the Influenza Commission² and the Research Laboratory workers that the problems³ to be investigated in New York City should be the following:

1. A study of the microörganisms of the upper respiratory tract in "health," in "common colds" and in "influenza." This investigation should include the diligent search for an epidemic microbial strain in influenza cases. Both filtrable and unfiltrable microbes were to be considered

2. A continuation of our studies on hemoglobinophilic bacilli (group of the influenza bacilli) for the purpose of establishing the relationship of the influenza bacillus to both pandemic and sporadic cases of influenza.

3. A study of the permanence of type characteristics of influenza bacilli in persons after recovery from respiratory infections. It was considered of the utmost importance to establish as accurately as possible the stability of types.

¹ A series of papers from the Research Laboratory, Department of Health, New York City, and one from the New York Hospital.

² The members of the commission are Drs. M. J. Rosenau, G. W. McCoy, Lee Frankel, A. S. Knight, E. O. Jordan, W. H. Frost and W. H. Park.

³ The studies are a part of the investigation on the etiology and the prevention of the acute respiratory diseases being carried out in Boston, Chicago, Washington and New York under a grant from the Influenza Commission Fund of the Metropolitan Life Insurance Company.

4. A study of the incidence of common colds, "influenza," and pneumonia, following the controlled use of a vaccine made up of different types of bacteria claimed to be the cause of acute respiratory diseases.

In carrying out this work, a large part of the material for the first two problems was collected from employees of the Metropolitan Life Insurance Company who volunteered their aid. The rest of the material was collected from laboratory workers and from cases of "influenza" in the hospital for contagious diseases. The preventive value of the vaccine was tested upon 1600 of the employees of the Metropolitan Life Insurance Company.

In arranging the work for the first problem, the investigation of the incidence of microbes was planned to include enough normal cases so as to have for study a sufficient number of persons who would later develop acute "colds" or "influenza" in order to obtain significant comparative curves of microbial incidence beginning in pre-disease stages and continuing throughout the attack; also to note the effect, if any, of vaccination upon the subsequent microbial content of a case. Our plans were changed, however, because of an early outbreak of acute respiratory infections which were diagnosed shortly after as influenza. In our opinion there is considerable doubt as to the influenzal nature of many of these cases. Notwithstanding our large force of workers, we were unable to investigate these and at the same time study normal cases as fully as we had planned. We were afraid to postpone study of the new cases as the outbreak might stop and we should then have had no acute influenza cases for study. We concentrated, therefore, on a mass of "influenza" cases, hoping, in spite of our previous failure, to demonstrate a common microbial strain whether filtrable or non-filtrable.

In the first paper we attempt to show the value of different technical methods in the demonstration of the different species of microorganisms from the upper respiratory tract and to give the average comparative incidence of groups of microorganisms demonstrated by these methods in health and disease. We lay stress on the methods of obtaining and handling the material.

In the succeeding papers in this number of the Journal and other papers which will appear later, are reported the studies made on the isolations from colonies of the various species appearing on the original plate cultures made directly from the case or from inoculated mice. The purpose was the search for a common "outbreak" or epidemic strain among the cultures isolated.

In two additional papers in this number are given respectively: A brief history of two cases of accidental infection with the influenza bacillus and the persistence of type characteristics in the inoculated persons; The results of vaccines on the prophylaxis of respiratory inflammations.

DISCUSSION

The chief object of all of these investigations is to demonstrate a common "epidemic" or "outbreak" strain among the groups of microbes claimed to have caused epidemics. As much as we were able, we employed methods that might best demonstrate these groups. We continued to bear in mind, however, that the methods we used might also demonstrate in small numbers hitherto undescribed organisms that might have an etiologic relationship to these infections. In spite of this, we decided to study closely only the more dominant types that were demonstrated by our procedure, simply recording the minority groups as determined by colony appearances and the examination of stained specimens from these colonies. We recognized that our methods of collecting material did not take into account the possibility of the primary exciting factor growing chiefly beneath the surface of the mucous membrane. We believe we are justified in assuming the probability that such a specific microbe would predominate or at least be prevalent upon the surface of a localized area of mucous membrane during some part of the early stages of the infection. If this microbe were an aerobe capable of growing on the culture media employed, selected fishings from ten colonies of the dominant types appearing on these media should give a majority of cultures obtained from the epidemic cases having no fundamental differences.

The results of our studies indicate that of the different groups of microorganisms isolated by our procedure, all had the pecu-

liarity that each group was an assemblage of many types. This was equally true for the pneumococcus, the influenza bacillus, the green-producing streptococci and the hemolytic streptococci. We obtained no evidence of the existence of a common filtrable organism. We accepted as the most delicate test of identity of types in strains the presence of specific agglutinable and agglutinogenic substances as determined by direct agglutination and agglutinin absorption methods. Biochemical characteristics are insufficient for such a determination; at most they divide a species only into the broader groups. In our isolation work, we are aware that the number of colony fishings may not have been enough in every group of bacteria to rule out a common strain. In a study of case incidence alone, some groups may be ruled out as implicated in the epidemic; such as staphylococci, Gram-negative cocci and hemolytic streptococci. More work could be done on pneumococci, green streptococci, indifferent streptococci and some of the minority groups with a high case incidence. The question of the relationship between the bacterial types used in vaccination and the microbial strains obtained from the throats of the vaccinated remained mostly unanswered. The specific strains of organisms used in the vaccines were not found to any extent in either normals or the diseased. It is a curious fact that in all but one instance the fixed types of pneumococci, as found in eleven of all the cases examined, occurred among the unvaccinated influenza cases. This loses significance, however, in the light of the fact that only 6 of our cultured influenza cases had received vaccine and 42 had not.

The evidence of immunological response to the vaccine was, as might be expected from the above findings, apparent only in the lessened incidence of pneumonia. The percentage of colds was as great among the vaccinated as among the unvaccinated. The pneumonia incidence was much less. The greater multiplicity of types of microbes believed to be capable of exciting common colds over those usually exciting pneumonia, is possibly the explanation of the apparent uselessness of the vaccines employed in this series in preventing minor respiratory infections while apparently affording considerable protection against pneumonia.

STUDIES ON ACUTE RESPIRATORY INFECTIONS

I. METHODS OF DEMONSTRATING MICROÖRGANISMS, INCLUDING "FILTRABLE VIRUSES," FROM UPPER RESPIRATORY TRACT IN "HEALTH," IN "COMMON COLDS" AND IN "INFLUENZA" WITH THE OBJECT OF DISCOVERING "COMMON STRAINS"

ANNA W. WILLIAMS, MARY NEVIN AND CAROLINE R. GURLEY

ASSISTED BY ALICE MANN, HELENA HUSSEY AND FLORENCE BITTMAN

THE CHOICE OF METHODS

In order to decide what methods we should use to demonstrate the microörganisms that might have a relationship to acute infections of the upper respiratory tract, we first investigated those previously reported for the demonstration of such organisms. We were unable, however, in the large majority of the reports to find methods given in enough detail to use them as standards for comparison. Such questions as the following, for example, were usually treated vaguely. Just how and from what areas the material for examination was collected and at what stages of the disease? What definitely described culture media were used and how were they inoculated? How were the resulting cultures handled in order to demonstrate strain relationship?

From the standpoint of arranging methods, we divided the microörganisms for which claims had been made into two general groups, namely, aerobic and anaerobic microbes, subdividing each into filtrable and non-filtrable microbes. As the most vigorous claims had been made for certain aerobic non-filtrable microbes and for anaerobic filtrable ones, we arranged our methods with special reference to these. We divided the aerobic non-filtrable organisms further into four general groups; namely, Gram-positive cocci, Gram-negative cocci, hemoglobinophilic bacilli and "others." We concluded that one of the nearest approaches to a standard method for demonstrating filtrable organisms from the upper respiratory tract was that reported by

Foster (1) (chiefly Noguchi technic). For this reason and because we wished to corroborate his work, we decided to use his method as well as modifications of it.

In regard to demonstrating the aerobic non-filtrable microbes from the mucous membranes of the upper respiratory tract, although no comprehensive method of procedure was found that was described fully enough to be used, we decided that certain culture media with methods of inoculating them might be considered standard. Among these were Brown's (2) technic for the demonstration of Gram-positive streptococci and Avery's (3) medium for hemoglobinophilic bacilli.

In order to decide what other media to use, we had to determine first how we were to collect our material. This brought us to the question of how localized beginning "colds" are and when, if ever, we might expect to find the primary exciting microbe as the dominant organism on the surface of this localized area. That the specific organism may *not* be found as predominating at any stage on the surface of the mucous membrane in certain infections of the upper respiratory tract, is certain unless it be grown on a special medium. Such a medium should inhibit only the growth of the "normal" flora which may grow on the mucous membrane surface more rapidly and abundantly than some more recently implanted organisms (whether pathogenic or not).

However, it was taken for granted that in many cases the primary exciting organism would grow, if only for a short time, at or near the beginning of the infection as a dominant on the area first infected; also that if a sterile swab were rubbed over this area and brought at once in the right kind of contact with supposedly favorable mediums, representative growth might be obtained.

Our plan of procedure for collecting our material and in making our first cultures is tabulated as follows:

Outline of technic for collection of material and for first cultures

- A. A sterile cotton swab (on wire, thinner for nose, thicker and protected by West tube for nasopharynx) is rubbed over surface of mucous membrane of:

- a. Nostrils and nasopharynx in rhinitis.
 - b. Nasopharynx and tonsils in pharyngitis, tonsillitis and laryngitis;
 - c. All three areas in "normal cases."
- I. Each swab is then rubbed over a small area on surface of plate 1, (see below) then on plate 2 and then rubbed up in II. With a platinum loop some of the material on plate 1 is transferred to plate 3 and plate 4 and may be used for direct smears. Material on all plates is then streaked out with platinum loop in "sun-burst" pattern. Plates are as follows:
- Plate 1. Vitamine-blood-pour-agar (4). For general enriching medium especially good for meningococcus.
 - Plate 2. Oleate blood agar (3). For influenza bacilli and other Gram-negative organisms.
 - Plate 3. Veal blood-drop agar (4). As a surface dilution plate, especially good for microscopic colony diagnosis of influenza bacilli and some Gram-negative cocci.
 - Plate 4. Egg-yolk agar (5). For special organisms.
- II. Tube of Ringer's solution (or pneumococcus broth (6) or 0.8 per cent salt solution) is inoculated with the swab. This suspension of material is used in appropriate dilutions for the following cultures:
1. Shake serum agar semi-solid tube for zone growth.
 2. Shake blood-pour agar plate (Brown's technic) for types of streptococci.
 3. Blood poured agar plate (Brown's formula) for surface comparison with Smith and Brown's plates and for colony diagnosis of pneumococcus.
 4. Mouse inoculation (peritoneum) for pneumococcus.
- B. Washings with Ringer's solution, 80 cc. (or salt solution, from 10 cc. up to 100 cc.) from naso-oral mucous membranes.
- I. Direct cultures.
1. Aerobic—same as A except II 4.
 2. Anaerobic semisolid horse serum tube.
 3. Anaerobic cooked meat medium.^a

^a Cooked meat medium: 500 grams of chopped beef to 1000 cc. distilled water, brought to the boiling point. This is strained through cheese cloth and the beef

II. Filtrates—chiefly under anaerobic conditions.

1. Foster's technic (Noguchi's) (1).
2. Cooked meat medium.
3. Egg yolk (5).
4. Inactivated horse serum glucose agar.^b
5. 0.2 per cent glucose agar plus rabbit kidney.^c
6. Veal blood-drop agar plate.
7. Veal blood-drop agar slant.

The clinical data we recorded are the following: In normal cases the date of taking of culture, type and date of previous cold, number of colds a year, subsequent colds, whether inoculated previously with special vaccine. In abnormal cases in addition—time ill at date of first culture, type of cold—divided into rhinitis, pharyngitis, tonsillitis, influenza, total days ill, severity of sickness, highest temperature, possible sources of human contagion, contributing causes and in the influenza cases in addition the leucocyte incidence.

In each of a series of representative cases after obtaining material on the swab, it was decided to wash the nostrils and throat according to Foster's method and our modifications of this method. The washings were to be used for the filtration experiments and for direct aerobic and anaerobic cultures. Several methods of obtaining washings were tried besides that described by Foster. In the majority of the cases (outside of those in which we used strictly the methods recommended by Foster) we employed the following method:

distributed into test tubes, about 1 cm. in each. To the bouillon is added 0.5 per cent NaCl and 1 per cent peptone; it is adjusted to a pH value of 7.9 to 8.0 and autoclaved for thirty minutes at 15 pounds pressure. About 10 cc. is added to the tube containing the meat, a layer of albolene, about $\frac{1}{2}$ inch, floated on top and then sterilized in the autoclave at 15 pounds pressure for thirty minutes.

^b *Inactivated normal horse serum glucose agar:* (1) 2 per cent veal agar containing 1 per cent peptone, 0.5 per cent NaCl, 2 per cent glucose, pH value 7.6 to 7.8.

(2) Normal horse serum inactivated at 56°C. for two hours on three successive days.

(3) 0.1 per cent glucose veal infusion broth—pH 7.8. Equal parts of the inactivated serum and 0.1 per cent glucose broth were mixed and added to the glucose agar in the proportion of 2 parts of serum broth to 1 part of agar.

^c Shake tube of 0.2 per cent glucose serum agar, pH 7.6 to 7.8, containing rabbit kidney ground up in ascitic fluid.

A sterile nose syringe, 40 cc. capacity, was filled with Ringer solution and inserted into one nostril. The solution was gently forced through to the nasopharynx and as it ran out through the other nostril it was caught in a sterile funnel attached to a sterile rubber tube and thence into a sterile dish. Forty cubic centimeters more of the solution was used as a gargle and added to the first 40 cc.

In trying to decide the question of the primary localization of "colds," we had to consider whether in rhinitis the cold started definitely in one or both nostrils or in the nasopharynx; in pharyngitis, tonsillitis and laryngitis, whether in either of these places or in the nasopharynx also. In order to help us interpret the findings from any of these areas in disease, we determined to obtain material in a definite number of our normal cases, from the middle fossa and floor of each nostril, from the nasopharynx and from the tonsillar surfaces; and in our disease cases, to take from the area reported as first infected and always from the nasopharynx. The mediums decided upon for the first inoculations from the swab were "vitamin-blood-agar" (4) and pneumococcus broth (6). From the vitamin blood-agar, oleate plates were inoculated and then veal blood-drop plates and McCoy medium. From the pneumococcus broth tube, two 1:10 dilutions were inoculated into melted blood-agar plates according to Smith and Brown's technic. Also, a semisolid glucose serum agar tube was made and a surface blood-pour plate for comparison with the Smith and Brown plates and to aid in the identification of pneumococcus colonies.

In recording the results from aerobic non-filtered material the following card was used in the way indicated.

We indicated the amount of growth by the plus sign, making four pluses the maximum. We indicated comparative numbers of colonies tentatively diagnosed as belonging to the different groups of organisms given at the top, in percentages and checked these up later with the reports of individual fishing findings.

The method of further handling the aerobic plates was based on the supposition already indicated; namely, that the primary

Case 11
Aerobic A and B; Date, November 24, 1919

MEDIA	AREA	GRAM POSITIVE COCCI					B. INFLU-ENZAE		GRAM NEGATIVE COCCI			OTHERS			RELATIVE ZONE	AMOUNT OF GROWTH	REMARKS
		Hemolytic strept. β	Green strept. α	Pneumo-cocci	Staph.	Indifferent γ	Typical	Atypical	Meningo-coccus	M. Catarrhalis	Other	Large Gram-neg. bacillus	B. mucosus	Other			
Vitamin, I-1	NR				40									60*	±		* <i>B. segmentosus</i>
	NL				30									70*	±		* <i>B. segmentosus</i>
	NP	20						40					20		++		* <i>M. siccus</i>
	T	40											60		++		
Oleate, I-2	NR														—		
	NL														—		
	NP							50					50		++		
	T						?						100		++		
Veal blood drop, I-3	NR														—		
	NL														—		
	NP							10					90		++		
	T		50				?						50		++		
Egg yolk agar, I-4	NR														—		
	NL														—		
	NP												80		+		
	T							20							+		
Brown II ₂	NR														—		
	NL														—		
	NP	70						?					30		++		
	T	80									?		20		++		

Blood poured, II _s	{ NR NL NP T }	40	20	10	30	- - +++++
Semisolid, II _i	{ NR NL NP T }					- - +++++ +++++
Mouse perito- neum	Mixed from all Living					
Vitamin.....	Washing			5	30	+ + + + +
Oleate.....	from nose		50		50	+ + + + +
Blood drop.....	and		10		30	+ + + + +
Brown.....	throat		?		20	+ + + + +
Blood pour.....			?	10	30	+ + + + +

exciting microörganisms may be present as the predominating organism on the surface of the area primarily infected, at the height of a curve which follows the course of the disease and is highest soon after the appearance of symptoms. As we said in our introduction, we had to leave out of consideration the supposition of a minority disease curve. The great majority of our "cold" cases and some of our "influenza" cases had their initial examination the first day of the appearance of symptoms. In the other influenza cases, the examination was made within the second and third days. Consequently, if the above supposition were a fact and if we were using suitable culture media, the predominating type of colony would be that of the primary exciting organism; and in the influenza cases the majority of fishings from similar predominating colonies would yield cultures with like characteristics if they belonged to the epidemic strain.

Therefore, after recording the colonies in the manner just described, the plates showing the best growths of colonies belonging to any one group of organisms were passed on to those workers who were studying that particular group of organisms.

These workers fished ten or more of the most abundant variety of colonies. These fishings were studied as described in the succeeding papers in order to determine whether a common strain existed among them. The workers reported their results to the central group who checked up the original colony diagnosis with these later tests; consequently, the report in this paper of the relative incidence of specific groups of bacteria is reasonably well controlled.

The following technic was used by Nevin for the isolation and identification of the Gram-negative cocci:

The plates were examined under the microscope for characteristic gram-negative-like colonies. Slide agglutinations were made from the meningococcus-like colonies. From the colonies showing an agglutination in 1:10 with a known polyvalent meningococcus serum, fishings were made to serum glucose agar.^d These cultures were sent to the meningococcus group of workers for type classification.

^d *Serum glucose agar*: 1.3 volume of normal horse serum added to 2 per cent glucose, 2 per cent agar, pH 7.8.

Ten fishings each were made of the predominating Gram-negative coccus-like colonies to chocolate agar^e and after an incubation at 37°C. for from twenty-four to forty-eight hours they were examined microscopically. When a contamination was evident, the cultures were plated and purified.

For the final identification of the isolated Gram-negative cocci a series of fermentation tests were made. In the first two series of tests a liquid medium was usedⁱ while for the third and fourth series we used a solid medium.^g The fermentative action of each sugar medium was controlled by known cultures giving positive and negative reactions.

The filtration and further handling of the filtrates was carried on by two special groups of workers (headed respectively by C. R. Gurley and Mary Nevin).

Berkefeld filtration (Berkefeld N) was done by water suction (except during the last part of the work when a Censo-Nelson electric pump was substituted). The water suction represented a low degree of exhaustion, the pressure within the filtering flask being about 9 pounds.

Judging from the reports of Noguchi's work on poliomyelitis and those of the Mt. Sinai Hospital workers on encephalitis, the question of the kind of ascitic fluid is of primary importance. We have tested and discarded many samples. Those used had a specific gravity of from 1.015 to 1.017, occasionally of 1.014 and 1.018. Specimens containing an indication of bile-content were avoided.

The procedure of one group of workers (under Nevin) may be described in detail as follows:

^e *Chocolate agar*: 2 per cent glucose veal agar pH 7.8. 2.5 cc. citrated horse blood added to every 70 cc. of the agar—previously melted and cooled to 60°C. The temperature is then gently raised to 78°C. until an even chocolate color is produced. This is then tubed, slanted, and tested for sterility before use.

ⁱ *Liquid carbohydrate medium*: Hiss serum water, 1 per cent carbohydrate, 1 per cent Andrade indicator.

^g *Solid carbohydrate medium*: One-third volume of a 2 per cent agar (sugar free), one-third volume of veal infusion broth (sugar free), one-third volume of inactivated normal horse serum, 1 per cent carbohydrate, 1 per cent Andrade indicator.

Three cooked meat medium tubes were inoculated with 0.1, 0.5, and 1 cc., respectively, of the unfiltered nasopharyngeal washings. After one week's incubation these cultures were filtered through a Mandler filter and the filtrates inoculated in amounts varying from 0.5 to 3 cc. into three meat medium tubes, and three inactivated horse-serum glucose agar tubes. These cultures were kept under observation for six weeks. In no instance was a filtrable organism obtained.

The filtered nasopharyngeal washings were treated in the following manner:

Three serum glucose agar tubes and two meat medium tubes were inoculated with amounts varying from 0.5 to 3 cc.

The remainder of the filtrate (from 30 to 40 cc.) was centrifugalized for one hour, and the sediment inoculated in the same number of tubes of serum glucose agar and meat medium.

All cultures were carried on under anaerobic conditions.

The procedure of the other groups of workers (under Gurley) may be described as follows:

The filtrates were centrifugalized at high speed for fifteen minutes. From 11 to 12 cultures were made from each filtrate, 7 in the kidney ascitic medium and one each in the other mediums listed.

The kidney ascitic tubes were each inoculated with 1 cc. of the centrifugalized filtrate and about 0.5 cc. was used for the other media. Two or more controls were always carried through.

A sample of the recording cards used for the anaerobic work with the filtrates is shown from case 49.

After we had run through 10 normals and 10 acute rhinitis cases by our procedure as originally planned, we obtained some results that led us to make a few changes.

1. In practically no "normal" nostrils did we get growths from the upper fossae and very slight, if any, growths from the floor unless the swab went back to the nasopharynx. Whatever scanty growths occurred, consisted almost entirely of small "*diphtheroid*" bacilli belonging morphologically to the segmentosus group; and of white staphylococci.

Investigation of acute respiratory infection; Case 49

Culture work B

DATE	WASHINGS										REMARKS	FILTRATE				
	Anaerobic cultures											Anaerobic cultures				
	Semi-solid		Method	Number of tubes	Filtrate							Semi-solid		Method	Number of tubes	
	Number of tubes	Zone			Date	Filter test	Method	Cultures made		Amount received		Number of tubes	Zone			
								Number of tubes	Result							
11/21/19	1	—	Meat	3	No filtration							cc. 50	1	—	Meat H. ser. ag. N. asc. k. Gl. ser. sh. Gl. kid. sh. Egg	4 6 8 1 1 1

Further results from anaerobic cultures

COLONIES FROM WASHINGS						METHOD	TUBES FROM FILTRATE									
Inverted plates from semi-solid zone				Hydrogen plates			Growth									
Date	Number	Number fished	Character	Date	Number		Character	Date	Number of tubes	Turbid	Precipitate	Stained film			Plates from tubes	
												Cocci	Bacilli	Other	Num-ber	Fished
							H. ser. ag.	12/5/19	6	—	—					
							Meat		2	—	—					
							N. asc. k.	11/21/19	8	2	—	+	2*		1	No. gr
							Gl. s. sh.	11/21/19	1	—	—					
							Gl. k. sh.	11/21/19	1							
							Egg	11/21/19	1							
							Controls*		10				1			

* Same as in the N. asc. k. control tube.

Abbreviations:

H. ser. ag. means horse serum agar.

N. asc. k. " Noguchi kidney ascitic fluid medium.

Gl. ser. sh. " glucose serum agar shake.

Gl. kid. sh. " kidney glucose serum agar shake.

2. In practically none of our early acute "rhinitis" cases, were we able to get marked growths of any organisms from the upper parts of the nostrils. In the few where we did obtain growths, the organisms consisted chiefly of the two groups found usually in the "normals." From the anterior part of the floor the results were about the same in kind as from the upper part of the nostrils. When the swab was inserted farther back, the cultures obtained became similar in kind to those of the nasopharynx, but they were likely to be over-grown by staphylococcus.

3. In the nasopharynx, however, the increase in growth was marked. On closely questioning our succeeding cases in regard to first symptoms, the patients described them almost invariably as being "farther in, than the nostril." We came to the conclusion that the majority of our cases of rhinitis as well as pharyngitis would show more representative early cultures from the nasopharynx, consequently, although we did make many more nasal examinations, the majority of our swab collections were made only from the nasopharynx when taken early in the disease.

4. The aerobic plates from our washings (B) showed a relative predominance of green-producing organisms. If suitable dilutions (much higher than the 1:100 and 1:1000 dilutions planned as routine) were used for showing well separated colonies, all other groups fell out in the majority of cases and were present in very small numbers in others. On the vitamin blood agar there were overgrowths, consequently the making of these plates was temporarily dropped.

5. Both in "normals" and in the diseased, cultures from tonsils showed a much larger proportion of hemolytic streptococci than cultures from any other area.

6. Injections of A II directly into the mouse, gave no results so Ringer's solution was changed to pneumococcus broth and this was incubated over night.

7. From our filtrates in normal cases, we obtained no cultures; from our early rhinitis cases, neither cultures nor reactions in humans after inoculation. We filtered no more normal washings.

8. We obtained no evidence of zone or special anaerobic growth in our deep semisolid tubes, so they were temporarily discontinued.

RESULTS

1. From aerobic cultures of non-filtered material.

Although the cultural results were influenced greatly by the area from which the material was obtained, the effect of the different kinds of media was equally as marked. Thus on our vitamin medium we had abundant growths of all groups, so abundant, that in many cases we had overgrowths. On Avery's oleate agar, as was to be expected with an inhibiting medium, chiefly influenza bacilli and certain Gram-negative cocci and other Gram-negative bacilli grew. It is interesting that most strains of meningococci do not grow well on this medium, while most strains of gonococci and of *B. pertussis* do. On veal blood-drop plates, by our procedure, the proportion and size of influenza bacilli colonies were less, while in Brown blood pour plates the influenza group and all other groups, except the Gram-positive cocci, appeared usually in still smaller numbers. This was due, partly, to our manner of inoculating the material but in a series of cases where we put the swab from the case directly into the diluent (pneumococcus broth) and inoculated equal proportions of broth in each medium, our results were practically the same. The copy of one of our primary case records (p. 10) indicates these differences.

Nevin group of workers

2. From anaerobic cultures of non-filtered washings.

No filtrable organisms were found.

3. From anaerobic cultures of filtered washings.

In six out of the forty cases cultured in this manner, anaerobic organisms were isolated. Two cases yielded a Gram-positive non-hemolytic streptococcus, one other case a diphtheroid; two cases a Gram-negative coccus and one case a small Gram-positive coccus. In only one instance was the growth found in more than one of the inoculated tubes and in this instance only two showed evidence of growth. The controls remained sterile. Meat medium cultures of all of these organisms were filtered through Mandler filters. No growth occurred in any of the cultured filtrates.

No further work was done with these cultures because they seemed to be of no etiologic significance. On the contrary, they were regarded as contaminants, although all of the control tubes (2 meat and 3 horse serum glucose agar) in each case remained sterile.

Gurley group of workers

4. From anaerobic cultures of filtered washings. (Noguchi technic chiefly).

Growths were obtained in the anaerobic cultures of filtrates from 4 out of 10 consecutive cases of influenza. In all of these cultures, a minute coccoid organism differing somewhat morphologically in two of the cases, was found. These were all obtained from the filtrate of the nasopharyngeal washings but they could not be carried on for more than one culture generation. No animal inoculations were attempted.

Of 23 early rhinitis cases, one showed an anaerobic coccus which died out in the third culture generation; one other case, a coccus, probably anaerobic, which died out in the second culture generation. In 20 normal washings no organisms were found.

From the filtrates of 7 early "cold" cases of a type similar to that described by Foster and from three typical influenza cases, we were unable to infect 45 human volunteers, 20 of whom were students less than thirty years of age. In the method of collecting the material and doing the inoculations Foster's technic was followed as closely as possible.

MICROBAL DISEASE CURVE

In our attempts to demonstrate in a series of individual cases a microbial curve which extended from health throughout an attack of acute respiratory infection, we were not successful. It is true that in a few cases of "colds" which occurred among our normals, we had a new type of organism predominating during the attack and disappearing shortly afterwards. These organisms were different in each case. Only two cases that we had examined as normal came down afterwards with an attack diagnosed as influenza. In both cases new types of organisms

appeared but not as predominants and apparently not the same in both cases. In a few other cases diagnosed as influenza we were able to take successive cultures throughout the course of the disease but these cases all developed pneumonia and one case died, so the microbial picture was interfered with by secondaries.

COMPARATIVE INCIDENCE OF MICROORGANISMS FROM NASO-PHARYNX AS RECORDED IN OUR ORIGINAL RECORDS

In trying to indicate the changes which we found in "normals," "colds" and "influenza" in the incidence of the group of

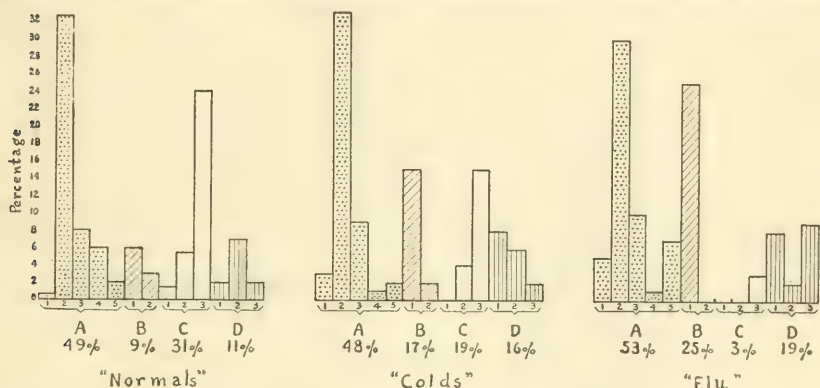


Chart Showing Comparative Incidence of Microorganisms by Groups

EXPLANATION OF CHART

A = *Gram-positive cocci*.

1. "Hemolytic streptococci" (Smith and Brown's beta type).
2. "Green producing streptococci" (Smith and Brown's alpha type).
3. *Pneumococcus* group.
4. *Staphylococcus* group.
5. "Indifferent streptococci" (Brown's gamma type).

B = *Hemoglobinophilic bacilli*.

1. "Typical."
2. "Atypical."

C = *Gram-negative cocci*.

1. *Meningococcus* group.
2. *Micrococcus catarrhalis* group.
3. *M. flavus*, *M. siccus* and others.

D = *Other microorganisms*.

1. "Large Gram-negative bacilli."
2. *Bacillus mucosus* group.
3. "Others."

microorganisms according to the chart headings, we obtained from our primary cultures the common incidence in each group by estimating the average percentages for each case on all the culture media used, adding these percentages in each individual group and dividing the sum by the total number of cases in that group. The following chart gives this common incidence in percentage for each group of cases.

Table 1 gives the case incidence in percentages for these groups of microorganisms.

TABLE 1

	GRAM-POS. COCCI						HEMOGLOBIN- OPHILIC BAC.	GRAM-NEG. COCCI			OTHERS		
	Total num- ber of cases	Hem. strep. β	Green strep. α	Pneumococ- cus	Staphylococ- cus	Indifferent γ		B. influenzae	Meningococ- cus	M. catarrh- alis	Others	Large gram neg. bacillus	B. mucosus
"Normals"	75	6	89	26	12	4	40	4	14	73	13	16	10
"Colds"	77	16	91	39	65	14	65	0	16	67	26	17	30
"Influenza"	48	27	100	50	10	44	92	0	2	29	44	4	79

INTERPRETATION OF CHART AND TABLE

In the first place it should be remembered that the chart and the table give no indication of the amount of growth. That was recorded on the original sheets (pp. 10 and 11) and may be summed up as follows: From the nasopharynx of the "normal cases," on the whole a very scanty growth was obtained. In the dilutions we employed, we often obtained no growths on our Brown plates, in fact, from some cases there was practically no growth on any plates, except on the vitamin-blood-agar and the oleate, where the growth was a scanty one. In the "colds," the growth on the whole was very abundant, though in some of the cases obtained in the first hours of the disease, the amount was scanty. In all of the influenza cases, the growth from the nasopharynx was very abundant.

In studying the comparative incidence in the types of clinical cases of the four groups of microorganisms as shown by the chart, we note that there are few differences if the groups are considered as a whole. We see simply that the Gram-negative cocci group drops somewhat in the "cold" cases and markedly in the influenza cases, while the influenza bacilli and the "others" progressively increase. When we study the subgroups, however, more differences appear. But we must take into account more minute colony characteristics in order to appreciate further differences.

Thus, among the Gram-positive cocci, the "alpha type" of streptococcus (green-producing streptococci) predominate in all three kinds of cases. But in the "normals" and "colds," this type varied in general colony characteristics in the different cases while in practically every influenza case the majority of green-producing colonies seemed to be of one type and of a type that had not appeared before, at least in any numbers. We labelled these colonies "minute green" and these are the colonies from which isolations were made for further study and comparison with the fishings of green-producing colonies which we had made from the "normals" and "colds." (See paper III of this series.)

The "beta type" of streptococcus shows a small increase when compared with group incidence, although in disease the increase over its appearance in normals is marked. It is significant that out of the 12 cases of colds in which hemolytic streptococci appeared in the cultures from the nasopharynx, 7 of the cases began as tonsillitis. The further study of the beta streptococci from this series of cases is to be reported later.

The pneumococci show a very slight increase in "colds" over the "normals" and in "flu" over the "colds." The further study of the strains is given in Paper II of this series. No common type was found. It is interesting that of the few fixed types found in the influenza cases, all occurred in the ten cases not inoculated with our vaccine (see Povitsky paper), but, as only 6 of the 48 cases in this series had been inoculated with the vaccine, this observation has little, if any, significance.

Since the staphylococci were infrequently demonstrated from the nasopharynx in disease, we decided to discontinue the study of the individual fishings.

Although there was a marked increase of the "indifferent streptococci" (Smith and Brown's gamma type) in "colds" and "influenza," over the percentage usually found in "normals," there were very few in the individual cases. However the increase occurring in "flu" case incidence indicates that a minority curve might be found that may be of significance.

That among these colonies diagnosed originally as "indifferent," that is, producing no changes on standard blood-agar-plates, some may be found on further study to be alpha streptococci type, is possible. We fished a number of these colonies from many cases and studied primary cultures in a general way hoping to be able to classify them further, but we had to discontinue their study because of the emphasis we were placing on majority incidence.

In regard to the hemoglobinophilic bacilli the most interesting point brought out in the chart, is that the atypical influenza bacilli (see Povitzky's paper) occur less commonly in the "cold" cases than in the "normals" and drop out entirely in the primary cultures from the influenza cases which were studied. This makes the increase in the typical influenza bacilli in the "influenza" cases very marked. The case incidence is 92 per cent. Of course, we show (Povitzky's paper) that there is no one serologic type in this series of cases, thus corroborating our earlier work.

In some of the our early "cold" cases, we found large numbers of *M. catarrhalis*. Chiefly for this reason and partly because claims had been made for Gram-negative cocci as an etiological factor, we started to study individual fishings of this group. We studied them for only a short time, however, owing to the fact that neither they nor practically any kind of Gram-negative coccus appeared in our "influenza" cases.

Although types of Gram-negative cocci may have a relationship to certain cases of "common colds," judging from the comparative incidence none had anything to do with the influenza cases in this series.

Among the "others," we recorded and fished colonies of large Gram-negative bacilli showing similar characteristics. These bacilli occurred in increased abundance over normals in "colds" and "influenza" cases with a corresponding increase in case incidence. We made many fishings from this type of bacillus but were unable to finish our studies on it. We have concluded that it may be an important factor in "common colds."

B. mucosus, we recorded because of its relatively abundant growths in certain cases in the "normals" and "colds" but we made no study of individual fishings. It dropped almost out of sight in the influenza cases. The last column in the Chart in the groups of "normals" and "colds" is a record of different organisms from individual cases, some Gram-negative bacilli, some Gram-positive bacilli which were different in the different cases. On the other hand, in the "influenza" cases, this record is due chiefly to "minute" delicate colonies appearing on our first vitamin-blood-agar plates in close proximity to colonies belonging to other groups. When we diluted the material further they dropped out. We attempted to isolate these organisms and obtained in the majority of cases a minute cocco-bacillary form of short chains and clumps. These died out quickly (we made the majority of our fishings on chocolate agar) and as they were in such great minority and we were studying majorities we discontinued their study.

CONCLUSION

The procedure for collecting and handling material as outlined in this paper is a more comprehensive one than has been given before for demonstrating the comparative incidence of the great groups of microorganisms that have usually been found in the majority of acute upper respiratory infections. Therefore, the colony fishings are more likely to be representative of a dominant type, if one exists.

The procedure has also demonstrated increased incidence in mucous membrane inflammations of certain minority groups due to an increase of one type of organism which might have a relationship to acute inflammations of the upper respiratory tract.

The négative results obtained by our procedure for demonstrating filtrable microbes, we consider an indication that the positive results already reported do not bear as wide an application as their sponsors infer.

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II. A STUDY OF THE SEROLOGICAL RELATIONSHIPS OF PNEUMOCOCCI FROM THE UPPER RESPIRATORY TRACT WITH SPECIAL REFERENCE TO COMMON COLDS AND INFLUENZAL CONDITIONS

GEORGIA M. COOPER, LUCY MISHULOW AND NINETTE E. BLANC

From the Bureau of Laboratories, Department of Health, New York City

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This study forms a part of a general investigation by the Bureau of Laboratories and the Influenza Commission of the Metropolitan Life Insurance Company of the flora of the respiratory tract in normal and pathological conditions. The purpose of this section of the work was to determine whether any one variety of pneumococcus would be encountered more frequently in epidemic inflammatory conditions resembling influenza than in those of the nature of ordinary colds and in the normal controls. If, in a wave of common colds or influenzal-like inflammations, a dominant variety could be demonstrated in the majority of cases it would suggest strongly that the pneumococcus strain in question was the primary causative agent of the outbreak in which it was prevalent. The presence of a single type of a pathogenic variety in the individual case may indicate, however, that it is either the primary agent or one of the secondary invading bacteria; or that it may be only an accidental parasitic variety. The problem was, therefore, to determine the similarities or dissimilarities among the various strains isolated from the nasopharynx of normal individuals, and of cases of rhinitis, pharyngitis, influenza and broncho-pneumonia. In all instances the nasopharyngeal material examined was the same as that from which the other species of organisms were isolated by the different workers engaged in this general investigation. The methods employed for the study of the similarities and dissimilarities of

the pneumococcus strains were those of agglutination and agglutinin absorption.

The study of the strains was based on the facts already known concerning the differentiation of the pneumococci. These facts may be briefly reviewed as follows. Park and Williams (1) classified *Streptococcus mucosus* as a definite variety of pneumococcus, which variety is now known as *Pneumococcus mucosus* or Pneumococcus type III. Collins (2), resorting to agglutination and agglutinin absorption with monovalent rabbit serums, found specific relationships among members of this mucosus group and group relationships with other varieties of pneumococci. Cole (3) and Hanes (4) also demonstrated marked cross agglutination among members of this group. Neufeld and Händel (5) also found specific types of pneumococci, which types were influenced only by their own antisera. These observations were confirmed and extended by Cole (3) and by Dochez and Gillespie (7) who showed that many pneumococci could be placed in two distinct serological types which they termed type I and type II. They defined type III as a distinct morphological variety and placed the strains which could not be classified with the above types in a heterologous group of strains termed group IV.

Pneumococci giving a partial or delayed agglutination with type II serums were later defined by Avery (7) as atypical type II or subgroup II strains. According to him, type II serum protected mice against lethal doses of the majority of these strains. He classified ten strains in two distinct groups subgroup A and subgroup B and a third heterologous group subgroup X. Subgroup A removed from type II serum the agglutinins for other members of that group but not for subgroup B. The subgroup B acted vice versa. However, neither of these removed the specific agglutinins for type II strains. With monovalent rabbit serums for the members of the subgroups, he found that subgroup A strains gave marked agglutination with antisera for members of that subgroup; that subgroup B strains agglutinated with subgroup B antisera but there was no cross reaction between these subgroups. Typical type II strains gave no reaction with the antiserum of either subgroup. Stillman (8) following the methods

of Avery classified 204 strains in twelve subgroups, the subgroups II-A and II-B of Avery and ten others subgroups II-C, D, E, F, G, H, J, K, L and M. The attempt to demonstrate definite subgroups among the strains provisionally classed as group IV, is considered later.

The literature on the incidence of the types of pneumococci in pneumonia is very extensive. The incidence of the different types in normal individuals including healthy contact carriers, has also been studied in detail but there is little information available on the occurrence of types in other conditions. Park and Williams (1) in 1905 investigated the pneumococci isolated from inflammations of the upper respiratory tract. At that time the separation of types had not been completed and correlation of their groups with those now recognized is not possible. Valentine (9) investigated the incidence of types of pneumococci in common colds with a view to their being a possible source of contagion for lobar pneumonia. She found pneumococci in forty-three out of sixty-five cases of common colds. The incidence of types was as follows: Group I, two cases; group II, two cases; group III, four cases; and group IV, thirty-five cases. Clough (10) published a study in which a few conditions other than pneumonias were included, but for the most part the conditions investigated by her do not coincide with those studied by us. In five cases of chronic bronchitis she found three type IV strains and two strains classed as miscellaneous. In two acute bronchitis cases, one strain was an atypical type II and the other a type IV.

Of 138 cases cultured by us, pneumococci were found by the methods employed, to be present in fifty-three. The criteria adopted by us for identification of an organism as a pneumococcus were typical morphology and bile solubility. All of the cultures fermented inulin, but a strain would not have been discarded had the ability to ferment this carbohydrate been lacking. In one instance a culture was lost before typing was completed; in five the pneumococci present were not isolated in pure culture because the plates were overgrown by *B. subtilis* or *B. proteus*. In these instances, it was inferred that pneumococci were present from the discovery of typical Gram-positive encapsulated diplococci in the

smears and from the bile solubility of the cocci obtained from the peritoneal washings of the injected mice.

Strains were obtained, therefore, from forty-seven cases. These strains were then tested with serums of the three types. For the identification of the fixed types, undiluted antipneumococcus horse sera were employed and the tests were incubated at 45°C. Of the forty-seven strains, nine were found to belong to the fixed types as shown by the prompt reaction with one or the other serum. These nine were distributed as follows: Type I pneumococci were isolated from two normal cases and one influenza; type II from two influenza cases; type III from four cases—one chronic rhinitis, one acute rhinitis, one pharyngitis and one influenza complicated by broncho-pneumonia. From the last case, a pneumococcus of subgroup IIB was isolated as well as pneumococcus type III. These results are included in the summaries (tables 17 and 18) and are discussed later in connection with the findings from further study of the other strains which did not fall into the fixed types.

The thirty-eight remaining cultures were further tested with type serums. The technic employed was as follows:

The stock cultures were maintained on blood streaked glycerin veal agar. Beef broth adjusted to a reaction of pH 7.6 to 7.8 was used as the medium for the growth of the agglutination antigen. Preliminary transplants were made into this broth and were incubated for eighteen hours to twenty-four hours. From these cultures final transplants were made into broth and these were incubated about eighteen hours. These cultures were tested for bile solubility to detect contaminations. The broth cultures according to their turbidity were diluted to 1 in 2 or 1 in 4 with salt solution to secure very thin suspensions. The dilution gave more clear cut results and the lessening of the color of the broth was advantageous. The tests were made by mixing 0.5 cc. of serum dilution and 0.5 cc. of diluted broth culture; these mixtures were incubated at 55°C. for two hours. Agglutination after incubation at 55°C. was found to be stronger than at 45°C. After the two hour incubation a preliminary series of readings was made. Then the tests were placed in the ice-box over night. In the morning the readings were again made. These were stronger than those after two hours' incubation, were clear cut and apparently not less specific than the earlier readings. They are recorded in the tables.

The agglutination tests of the thirty-eight strains with the diluted type serums are given in table 1. As controls, representative strains of type I, II and III were included, also several subgroup II cultures.¹ Fifteen strains were found which were agglutinated slowly or partially by the type II serum. It is interesting to note that with the majority of these fifteen strains, the reaction with the serum was not perceptible at the two hour reading and became apparent only after the tests had stood in the ice-box over night. A similar delayed agglutination occurred with several of the known control strains. Six of the fifteen strains have been definitely classified as belonging to subgroup II. These relationships are given below in the discussion of the results obtained by agglutinin absorption methods. Nine of the fifteen were found to be different from any of the subgroup II strains which were available as controls. These may belong to the subgroup II, but since a complete set of the subgroup strains already differentiated were not available for comparison, it would have been necessary to resort to protection tests for a final determination. This was not done and the strains are classified provisionally in the heterologous group IV. In the charts, they are designated as IV^u to call attention to the fact that they are agglutinated to some extent by type II serum. Eleven strains were slightly agglutinated by all the type serums. They are given in the table as IV*.

Blake (11) and Clough (10) encountered strains reacting similarly to the eleven just described. Clough does not include these among the group IV strains but terms them miscellaneous. Twelve of the strains studied by us did not agglutinate in the fixed type serums. These conform definitely to the criterion accepted for group IV strains and are tabulated as IV without further distinguishing symbol.

The relationships among the thirty-eight strains discussed above, were further studied by agglutination tests with monovalent antipneumococcus serums obtained from rabbits.

Serums were prepared for twenty-two strains.

¹ We are indebted to Dr. Avery for the subgroup II cultures.

Agglutination with antipneumococcus serums types I, II and III (horse)

[illegible]

TABLE 1—Continued

CASE NUMBER	EXAMI- NATION NUMBER	STRAIN NUMBER	TYPE I SERUM						TYPE II SERUM						TYPE III SERUM						CONTROLS	
			1:5	1:10	1:25	1:50	1:100	1:200	1:300	1:5	1:10	1:25	1:50	1:100	1:200	1:300	1:5	1:10	1:25	1:50		1:100
Controls:																						
		Type I.....	+	+	+	+	±	±	×	×	-	-	-	-	-	-	×	×	-	-	-	-
		Type II.....	×	-	-	-	-	-	-	+	+	+	±	±	×	×	×	×	-	-	-	-
		Type III.....	-	-	-	-	-	-	-	×	-	-	-	-	-	-	±	+	+	-	-	-
		Sub II A.....	×	-	-	-	-	-	-	+	+	+	±	×	-	-	-	×	-	-	-	-
		Sub II B.....	×	-	-	-	-	-	-	+	+	+	+	±	×	-	-	-	-	-	-	-
		Sub II E.....	-	-	-	-	-	-	-	±	±	×	-	-	-	-	-	-	-	-	-	-
		Sub II G.....	±	×	-	-	-	-	-	+	+	±	±	×	-	-	×	-	-	-	-	-
		Sub II H.....	-	-	-	-	-	-	-	±	±	×	-	-	-	-	-	-	-	-	-	-
		Sub II J.....	±	±	±	×	-	-	-	+	+	±	±	×	-	-	±	×	-	-	-	-
		Sub II M.....	±	±	±	×	-	-	-	+	+	±	±	×	-	-	±	±	×	-	-	-

Tests incubated for two hours at 55°C. Read after overnight in ice-box.

Symbols: + = complete agglutination; ± = marked; × = slight; - = no reaction.

To obtain serums sufficiently potent for agglutinin absorption tests, it was necessary to give the rabbits eight to twenty-five doses, about eighteen being the average. The dose was increased gradually from a dose equivalent to the sediment obtained by centrifugalization of 5 cc. of a broth culture, to that obtained from 50 cc. or 100 cc. In most instances living unheated cocci were injected from the beginning. Inoculations were given on three or four consecutive days and were followed by rest periods of three or four days. On the last day of the rest period trial bleedings were made. If the sera did not prove to be sufficiently potent, the injections were continued; if potent, the rabbits were bled to death on the fifth or sixth day after the last inoculation.

Every effort was made to obtain the highest possible titer. In some instances, a titer of 1:200 was the highest obtainable. Serums less potent than this were not used.

The results of the agglutination tests with these serums are given in table 2. The most noticeable feature is the small amount of cross agglutination with strains heterologous to the serums. This, however, was not unexpected as it corresponds with the results reported by others. Dochez and Gillespie (6) using eight monovalent serums found no relationship among fourteen group

TABLE 2
Agglutination with monovalent antipneumococcus serums (rabbit)

CASE NUMBER	EXAMINATION NUMBER	STRAIN NUMBER	CONDITION	DAYS ILL*	ISOLATED FROM	STRAIN NUMBERS OF MONOVALENT SERUMS																											
168 V	1	1	Influenza and pneumonia	22 (?)	m†	700†	168-V-1	166-II-1	90-III-1	158-I-1	17-I-1	72-II-4	69-II-1	57-I-5	94-I-5	76-I-1	134-I-1	165-I-1	23-I-4	38-II-2	52-I-1	130-I-1	2-II-1	71-I-5	86-I-6	156-I-1	160-I-1	163-I-1					
166 II	1	1	Influenza	7	m	300																											
107 I	1	1	Pharyngitis	3	m	200																											
135 I	1	1	Influenza	3½	m	300																											
157 II	1	1	Influenza and pneumonia	8	m	200																											
90 III	1	1	Influenza	4	m	700																											
158 I	1	1	Influenza	2	m																												
134 I	1	1	Influenza	6	m																												
17 I	1	1	Influenza	1	m	pl(†)																											
161 I	1	1	Rhinitis	1	m																												
72 II	4	4	Influenza and pneumonia	1	m																												
89 II	1	1	Normal	—	m																												
57 I	5	5	Rhinitis	2	m																												
94 I	5	5	Normal	—	m																												
82 I	1	1	Rhinitis	1	m																												
76 I	1	1	Normal	3	m																												
154 I	1	1	Influenza	—	m																												
165 I	1	1	Influenza	1	m																												
18 II	1	1	Rhinitis and pharyngitis	4	m																												
34 II	1	1	Rhinitis and pharyngitis	1	pl																												
39 II	1	1	Normal	—	m																												
69 II	1	1	Rhinitis	4	m																												
78 II	1	1	Normal	—	m																												
99 I	2	2	Rhinitis	1	m																												
	1	1	Rhinitis	6	m																												

IV strains. Cole (3) also found his group IV strains to be distinct varieties. Olmstead (12) in the preliminary study of 213 group IV strains reported that twelve subgroups were indicated by the direct agglutination. The data giving the methods by which the strains were classified as belonging to group IV are not given in detail and it is very probable in the light of Stillman's (8) later work that some of the groups Olmstead described would now be classified as sub-groups of type II. At this point, it should be stated that all the sub-group II strains studied by us were on the basis of the preliminary tests, placed in group IV. The relation to type II was shown only when a more advantageous temperature and time was employed and known subgroup II strains were included for comparison.

In our series of strains, the absence of cross agglutination indicated at once that strains 57, 94, 165, 71, 86, 156, 160 and 163 were distinctly different one from the other and from all the others in the series. A few strains, nos. 18, 34 and 69, showed slight cross agglutination with several serums. It should be noted that these strains are among those which agglutinate also with all three type serums. The reactions with these strains varied considerably from time to time with the different antigens employed; the agglutinability seemingly was influenced by slight differences in growth and in the media. These cross-reactions were checked in some instances by agglutinin absorption. The results indicate that these strains may be considered as different one from the other and different from the other strains for which antisera were prepared.

In the instances where cross agglutination was marked, the specificity of the reactions was investigated by agglutinin absorption tests. The technic was as follows:

The centrifugalized sediment from 300 cc. to 600 cc. of broth culture was collected in salt solution and placed in a 15 cc. graduated tube and centrifugalized until the organisms were packed at the tip of the tube. Approximately equal masses were obtained from like amounts of broth, about 0.1 cc. being obtained from 300 cc. of broth culture. The supernatant fluid was decanted. The amount of serum and salt solution to be added was estimated so that the proportion of the mass of packed

organisms to the final volume of serum dilution was as 1:5, 1:10, or 1:20, according to the titer of the serum to be absorbed. The estimated amount of serum required was added to the packed organisms and then sufficient salt solution to make a final dilution of 1 in 12.5. In calculating the amount of salt solution to be added, an allowance was made for the salt solution in the packed organisms. This was assumed to be one-half of the mass which is evidently in excess of the amount actually present. This excessive estimate meant that the dilutions actually employed were somewhat lower than as recorded. This means that the absorption if present was somewhat greater, not less, than given in the recorded figures. As the actual amount of water present in the packed organisms could not be conveniently determined, an excessive estimate was desirable as it made the test more rigid. The organisms were thoroughly distributed in the diluted serums. The mixtures were incubated at 45°C. for three hours. During this period the tubes were shaken frequently to obtain a greater contact with the serum. The mixtures were placed in the ice-box over night. In the morning they were centrifugalized and agglutination tests were made with the supernatant fluid, as well as with the unabsorbed serums as a control. The optimum absorbing dose was determined by treating a serum with its homologous organism; the dose sufficient to remove all the specific agglutinins was the standard for this serum. In a few instances where the results seemed sufficiently conclusive, incomplete absorptions are recorded. In most instances the serum was also treated with a culture which gave no agglutination with the serum, as a control of the absence of non-specific mass absorption.

The results of the agglutinin absorption tests with monovalent rabbit serums are given in tables 3 to 16. The subgroup IIA control strain removed the agglutinins from the antiserum for strain 168-V-I (table 3). A typical type II strain did not agglutinate, however, with this serum and when used in a mass equal to that employed for the other strains did not reduce the agglutinins for the strain homologous to the serum. This result corresponds with the findings by Avery and Stillman with reference to the agglutination reaction of typical type II strains with subgroup II sera. Strain 168-V-I was isolated from an influenza case complicated by broncho-pneumonia. It was classified as a member of subgroup IIA.

TABLE 3

*Agglutinin absorption of serum 168-V-1**

STRAINS	AGGLUTINATION, UNABSORBED SERUM								ABSORBED BY	AGGLUTINATION, ABSORBED SERUM								CONTROLS	
										Absorb- ing strain	Serum strain								
	1:25	1:50	1:100	1:200	1:300	1:400	1:500	1:600			1:700	1:800	1:25	1:50	1:100	1:200	1:300		1:400
168-V-1*	+	+	+	+	±	±	±	×	-	168-V-1	-	-	-	-	-	-	-	-	-
Sub. II-A	+	+	+	+	+	±	±	±	×	Sub. II-A	-	-	-	-	-	-	-	-	-
Type II	-	-	-	-	-	-	-	-	-	Type II	-	-	+	+	+	±	±	×	×

* The table reads from left to right, thus strain Sub. II-A is followed by the symbols of the degree of reaction with serum 168-V-1. In the next column ("absorbed by") the number is repeated to show that this strain was used for absorption and to indicate that the symbols immediately following, under the heading "absorbing strain" is the degree of agglutination by serum 168-V-1 after absorption by culture Sub. II-A. All the symbols under the heading "serum strain" refer to the agglutination of strain 168-V-1 by the serum after absorption by the strains as noted under the heading, "absorbed by."

In this table and succeeding table the first strain is homologous to the serum and the symbols under "absorbing strain" and under "serum strain" are duplications, as in these instances the serum strain is the same as the absorbing strain.

TABLE 4

Agglutinin absorption of serum 166-II-1

STRAINS	AGGLUTINATION, UNABSORBED SERUM							ABSORBED BY	AGGLUTINATION, ABSORBED SERUM							CONTROLS	
									Absorb- ing strain		Serum strain						
	1:25	1:50	1:100	1:200	1:300	1:400	1:500		1:25	1:50	1:100	1:25	1:50	1:100	1:200	1:300	
166-II-1	+	+	+	±	×	—	—	166-II-1	—	—	—	—	—	—	—	—	—
157-II-1	+	+	+	±	×	—	—	157-II-1	—	—	—	—	—	—	—	—	—
135-I-1	+	+	±	±	×	—	—	135-I-1	—	—	—	—	—	—	—	—	—
135-I-2	+	+	+	±	×	—	—	135-I-2	±	×	—	×	—	—	—	—	—
107-I-1	+	+	+	±	×	—	—	107-I-1	—	—	—	—	—	—	—	—	—
Sub. II B	+	+	+	±	×	—	—	Sub. II B	—	—	—	—	—	—	—	—	—
82-I-1	+	±	×	—	—	—	—	82-I-1	—	—	+	+	+	×	—	—	—
23-I-1	—	—	—	—	—	—	—	23-I-1	—	—	+	+	+	×	—	—	—

Strains 157-II-1, 135-I-1, 107-I-1 and a subgroup II-B control strain, absorbed the agglutinins for the homologous strain from antiserum 166-II-1 (table 4). Case 157, a fatal influenza and pneumonia, and case 166, a severe influenza, were brothers. Here, infection by contact was probable. On the other hand, the histories of case 135 an influenza and case 107 a pharyngitis, do not indicate that contact with each other or with cases 166 and 157 was likely. Strains from these four cases were classified as belonging to subgroup II-B. Case 157 was the one mentioned in the discussion of the fixed types from which type III was isolated as well as the subgroup II-B strain.

TABLE 5

Agglutinin absorption of serum 90-III-1

STRAINS	AGGLUTINATION, UNABSORBED SERUM								ABSORBED BY	AGGLUTINATION, ABSORBED SERUM								CONTROLS
										Absorb- ing strain		Serum strain						
	1:25	1:50	1:100	1:200	1:300	1:400	1:500	1:600		1:25	1:50	1:100	1:200	1:300	1:400	1:500	1:600	
90-III-1	+	+	+	+	+	+	+	×	90-III-1	-	-	-	-	-	-	-	-	-
Sub. II H	+	+	+	+	+	+	+	×	Sub. II H	-	-	-	-	-	-	-	-	-
II	-	-	-	-	-	-	-	-	II	-	-	+	+	+	+	+	×	-
76-I-1	-	-	-	-	-	-	-	-	76-I-1	-	-	+	+	+	+	+	×	-
38-II-2	-	-	-	-	-	-	-	-	38-II-2	-	-	×	+	+	+	+	×	-

Strain 90-III-1 from an influenza case was identified as a member of subgroup II-H (table 5).

Strains similar by agglutinin absorption were obtained from case 158 a very severe influenza and case 134, a moderately severe influenza (table 6). In these instances the probability of contact was remote.

From case 17, a rhinitis, and case 161, a very severe influenza, where contact was not indicated by the histories, similar strains were isolated. Three other strains giving slight cross agglutination failed to absorb the agglutinins for the homologous strain from anti-serum no. 17 and were classified as unlike strain 17 (table 7).

TABLE 6
Agglutinin absorption of serum 158-I-1

STRAINS	AGGLUTINATION UNABSORBED SERUM						ABSORBED BY	AGGLUTINATION, ABSORBED SERUM						CONTROLS	
								Absorb- ing strain		Serum strain					
	1:25	1:50	1:100	1:200	1:300	1:400		1:25	1:50	1:100	1:200	1:300	1:400		
158-I-1	+	+	+	+	±	×	158-I-1	-	-	-	-	-	-	-	-
134-I-1	+	+	+	-	±	×	134-I-1	-	-	-	-	-	-	-	-
161-I-1	±	×	-	-	-	-	161-I-1	-	-	-	+	+	+	±	×

TABLE 7
Agglutinin absorption of serum 17-I-1

STRAINS	AGGLUTINATION, UNABSORBED SERUM								ABSORBED BY	AGGLUTINATION, ABSORBED SERUM								CONTROLS		
										Absorb- ing strain			Serum strain							
	1:25	1:50	1:100	1:200	1:300	1:400	1:500	1:600		1:25	1:50	1:100	1:25	1:50	1:100	1:200	1:300		1:400	1:500
17-I-1	+	+	+	+	+	+	±	×	17-I-1	-	-	-	-	-	-	-	-	-	-	-
17-I-2	+	+	+	+	+	+	±	×	17-I-2	-	-	-	-	-	-	-	-	-	-	-
161-I-1	+	+	+	+	+	+	±	×	161-I-1	-	-	-	-	-	-	-	-	-	-	-
161-I-2	+	+	+	+	+	+	±	×	161-I-2	-	-	-	-	-	-	-	-	-	-	-
161-I-3	+	+	+	+	+	+	±	×	161-I-3	-	-	-	-	-	-	-	-	-	-	-
158-I-1	+	+	+	×	-	-	-	-	158-I-1	-	-	+	+	+	+	+	+	±	×	-
134-I-1	+	+	±	×	-	-	-	-	134-I-1	-	-	+	+	+	+	±	±	×	×	-
34-II-1	+	+	±	×	-	-	-	-	34-II-1	-	-	+	+	+	+	+	±	×	×	-

TABLE 8
Agglutinin absorption of serum 72-II-4

STRAINS	AGGLUTINATION UNABSORBED SERUM							ABSORBED BY	AGGLUTINATION, ABSORBED SERUM							CONTROLS	
									Absorb- ing strain		Serum strain						
	1:25	1:50	1:100	1:200	1:300	1:400	1:500		1:600	1:25	1:50	1:100	1:200	1:300	1:400		1:500
72-II-4	+	+	+	+	+	±	×	72-II-4	±	-	-	-	-	-	-	-	-
89-II-1	+	±	±	×	-	-	-	89-II-1	+	-	+	+	+	±	±	×	-
65-I-1	-	-	-	-	-	-	-	65-I-1	-	-	+	+	+	+	±	×	-
159-I-2	-	-	-	-	-	-	-	159-I-2	-	-	+	+	+	+	±	×	-
57-I-5	-	-	-	-	-	-	-	57-I-5	-	-	+	+	+	+	±	×	-
71-I-5	-	-	-	-	-	-	-	71-I-5	-	+	+	+	+	+	±	×	-

Although some cross agglutination occurred with the anti-serums for strains 72 and 89, no strains were found to be identical with either of these on absorption (tables 8 and 9).

From case 76 a normal control and case 154 a very severe influenza, where contact was improbable, similar strains were isolated (tables 10 and 11).

TABLE 9
Agglutinin absorption of serum 89-II-1

STRAINS	AGGLUTINATION, UNABSORBED SERUM						ABSORBED BY	AGGLUTINATION, ABSORBED SERUM						CONTROLS	
								Absorb- ing strain	Serum strain						
	1:25	1:50	1:100	1:200	1:300	1:400			1:25	1:50	1:100	1:200	1:300		
89-II-1	+	+	+	±	±	×	89-II-1	-	-	-	-	-	-	-	-
72-II-4	+	+	±	×	-	-	72-II-4	-	-	-	+	+	±	×	-
69-I-4	+	+	+	+	±	×	69-II-4	-	-	-	+	+	±	×	-
23-I-1	-	-	-	-	-	-	23-I-1	-	-	-	+	+	+	×	-

TABLE 10
Agglutinin absorption of serum 76-I-1

STRAINS	AGGLUTINATION, UNABSORBED SERUM										ABSORBED BY	AGGLUTINATION, ABSORBED SERUM													CONTROLS	
												Absorb- ing strain	Serum strain													
	1:25	1:50	1:100	1:200	1:300	1:400	1:500	1:600	1:700	1:800			1:900	1:25	1:50	1:100	1:25	1:50	1:100	1:200	1:300	1:400	1:500	1:600		1:700
76-I-1	+	+	+	+	+	+	+	±	±	±	76-I-1	±	±	-	±	±	-	-	-	-	-	-	-	-	-	-
154-I-1	+	+	+	+	+	±	±	±	×	-	154-I-1	±	±	×	±	±	×	-	-	-	-	-	±	±	±	-
38-II-2	-	-	-	-	-	-	-	-	-	-	38-II-2	-	-	+	+	+	+	+	+	+	+	±	±	±	±	-

The strains from case 23, a chronic rhinitis and case 38, an acute rhinitis, acted the same on absorption (tables 12 and 13). As case 38, a laboratory worker, assisted in taking the culture from case 23, transfer by contact cannot be excluded.

From case 52, a chronic rhinitis, case 130, an acute rhinitis, and case 169, a rhinitis and pharyngitis, strains were isolated which were alike. In these instances the possibility of contact

TABLE 11

Agglutinin absorption of serum 154-I-1

STRAINS	AGGLUTINATION, UNABSORBED SERUM							ABSORBED BY	AGGLUTINATION, ABSORBED SERUM							CONTROLS
	1:25	1:50	1:100	1:200	1:300	1:400	1:500		Absorb- ingstrain	Serum strain						
154-I-1	+	+	+	±	±	×	—	154-I-1	—	—	—	—	—	—	—	—
76-I-1	+	+	+	+	±	±	×	76-I-1	—	—	—	—	—	—	—	—
78-II-2	—	—	—	—	—	—	—	78-II-2	—	—	+	+	±	±	×	—

TABLE 12

Agglutinin absorption of serum 23-I-4

STRAINS	AGGLUTINATION, UNABSORBED SERUM							ABSORBED BY	AGGLUTINATION, ABSORBED SERUM							CONTROLS
	1:25	1:50	1:100	1:200	1:300	1:400	1:500		Absorb- ingstrain	Serum strain						
23-I-4	+	+	+	±	×	×	—	23-I-4	±	×	—	×	—	—	—	—
38-II-2	+	+	+	+	±	±	×	38-II-2	±	×	—	×	—	—	—	—
76-I-1	±	×	—	—	—	—	—	76-I-1	—	+	+	+	±	×	×	—

TABLE 13

Agglutinin absorption of serum 38-II-2

STRAINS	AGGLUTINATION, UNABSORBED SERUM						ABSORBED BY	AGGLUTINATION, ABSORBED SERUM						CONTROLS			
								Absorb- ingstrain	Serum strain								
	1:25	1:50	1:100	1:200	1:300	1:400		1:25	1:50	1:100	1:25	1:50	1:100	1:200	1:300	1:400	
38-II-2	+	+	+	+	±	×	38-II-2	—	—	—	—	—	—	—	—	—	—
23-I-4	+	+	+	+	±	×	23-I-4	—	—	—	—	—	—	—	—	—	—
76-I-1	+	±	×	—	—	—	76-I-1	—	—	—	+	+	+	+	±	×	—

was remote (tables 14 and 15). The strain from case 2, a normal control, showed a very marked cross agglutination with the antiserum (130-I-1), but when employed for the absorption of this antiserum, it only slightly reduced the agglutinins for the serum

TABLE 14
Agglutinin absorption of serum 52-I-1

STRAINS	AGGLUTINATION, UNABSORBED SERUM						ABSORBED BY	AGGLUTINATION, ABSORBED SERUM								CONTROLS	
								Absorb- ingstrain		Serum strain							
	1:25	1:50	1:100	1:200	1:300	1:400		1:25	1:50	1:100	1:25	1:50	1:100	1:200	1:300		
52-I-1	+	+	+	+	×	—	52-I-1	—	—	—	—	—	—	—	—	—	—
130-I-1	+	+	+	+	×	—	130-I-1	—	—	—	—	—	—	—	—	—	—
169-II-1	+	+	+	+	×	×	169-II-1	—	—	—	—	—	—	—	—	—	—
2-II-1	×	—	—	—	—	—	2-II-1	—	—	—	+	+	+	+	×	×	—
18-II-5	—	—	—	—	—	—	18-II-5	×	—	—	+	+	+	+	+	×	—

TABLE 15
Agglutinin absorption of serum 130-I-1

STRAINS	AGGLUTINATION, UNABSORBED SERUM										ABSORBED BY	AGGLUTINATION, ABSORBED SERUM										CONTROLS		
												Absorb- ing strain	Serum strain											
	1: 25	1: 50	1: 100	1: 200	1: 300	1: 400	1: 500	1: 600	1: 700	1: 800			1: 900	1: 25	1: 50	1: 100	1: 200	1: 300	1: 400	1: 500	1: 600		1: 700	1: 800
130-I-1	+	+	+	+	+	+	+	+	×	×		130-I-1	-	-	-	-	-	-	-	-	-	-	-	-
52-I-1	+	+	+	+	+	+	+	+	×	×		52-I-1	-	-	-	-	-	-	-	-	-	-	-	-
52-I-2	+	+	+	+	+	+	+	+	×	×		52-I-2	-	-	-	-	-	-	-	-	-	-	-	-
169-II-1	+	+	+	+	+	+	+	+	+	+		169-II-1	×	-	-	×	-	-	-	-	-	-	-	-
169-II-2	+	+	+	+	+	+	+	+	+	×		169-II-2	-	-	-	-	-	-	-	-	-	-	-	-
2-II-1	+	+	+	+	+	+	×	+	+	×		2-II-1	-	-	-	+	+	×	-	-	-	-	-	-
2-II-2	+	+	+	+	+	+	×	-	-	-		2-II-2	×	-	-	+	+	+	+	×	-	-	-	-
72-II-4	-	-	-	-	-	-	-	-	-	-		72-II-4	-	-	-	+	+	+	+	+	×	-	-	-

strain. The reverse absorption tests with the antiserum for strain 2-II-1 are reported in table 16. In this case the heterologous strains did not reduce the specific titer. These results indicate that the strain from case 2 was not similar to those from case 130, case 169 or case 52.

To summarize: Among the strains not belonging to the fixed types but which were covered by the monovalent serums available, were found six small groups and thirteen strains unrelated one to the other or to the groups just mentioned. This gives a total of nineteen types. Of these, one group and two separate strains belonged to the subgroup II classification. Five groups and eleven unrelated strains were provisionally placed in group IV. Of these, two groups and four unrelated strains belonged among those which gave slight agglutination with type II serums but were not further investigated as to whether they were true subgroups of type II. The strains of another group and one unrelated

TABLE 16
Agglutination absorption of serum 2-II-V

STRAINS	AGGLUTINATION UNABSORBED SERUM					ABSORBED BY	AGGLUTINATION, ABSORBED SERUM										CONTROLS
							Absorb- ing strain		Serum strain								
	1:25	1:50	1:100	1:200	1:300		1:25	1:50	1:100	1:25	1:50	1:100	1:200	1:300			
2-II-1	+	+	+	±	×	2-II-1	—	—	—	—	—	—	—	—	—	—	
130-I-1	+	±	×	—	—	130-I-1	—	—	—	—	+	+	+	×	—	—	
169-I-1	+	+	±	×	—	169-I-1	—	—	—	—	+	+	+	×	—	—	
52-I-1	+	±	×	—	—	52-I-1	—	—	—	—	+	+	+	×	—	—	
39-II-2	±	—	—	—	—	39-II-2	—	—	—	—	+	+	+	×	—	—	
57-I-1	—	—	—	—	—	57-I-1	—	—	—	—	+	+	+	±	×	—	

strain gave slight agglutination with all three type serums. Finally two groups and six unrelated strains comprised those which did not agglutinate at all with the fixed type serums.

There were, in addition, nine strains which were not similar to any of the strains for which there were antiserums. As no antiserums were prepared for these nine, the relationships among them could not be investigated. It is reasonable to suppose that further tests with these strains would yield results similar to those found among the strains for which antiserums were produced.

The results from this part of the investigation are collected in table 17 and are summarized with reference to type and pathological condition in table 18. From these data, the prevalence

of a particular type of pneumococcus in any of the conditions studied is not indicated.

In addition to the classification of strains from different cases, a brief study was made of strains isolated from the same case to determine whether several varieties of pneumococci would be found in the naso-pharynx. Colonies morphologically different as far as possible were chosen, varieties of contour, size and color being noted. The agglutination of the strains from individual cases with the antiserum homologous to one strain from each case are given in table 19. In other cases, a serum was considered homologous even if not actually prepared with a strain from a case, if the absorption of the serum by a strain of the case in question was complete. The results with serums considered homologous on the basis of absorption are given in table 20. In but one case was there encountered more than one variety of pneumococcus. In all cases where a subsequent examination of an individual was made, the same variety was isolated again. The instance in which two varieties were encountered has been noted.

For the most part, the fishings considered in tables 19 and 20 were made from a single collection of material. In a few cases, material for cultures was taken a second or third time after intervals as recorded in the tables. Attention must be called to the fact that in many instances the strains of pneumococci were obtained from mice injected with the original material. That by this method only one variety was recovered from a case was not surprising. It was to be expected that the mouse would serve in a selective manner; the most virulent variety would most likely be recovered in predominance or alone. However, where pneumococci were obtained from plates inoculated directly with the material from the nasopharynx, the results were the same as after mouse passage.

The results of previous workers as regards the fermentative reactions of pneumococci do not suggest that these reactions would be of value in the differentiation. Park and Williams (1) found some differences in mannite fermentation; about 70 per cent of their strains fermented this alcohol. All their mucosus

Tabular summary—Variety of pneumococcus from cases in relation to condition

NORMAL		CHRONIC RHINITIS		ACUTE RHINITIS		PHARYNGITIS	
Case number	Type	Case number	Type	Case number	Type	Case number	Type
36	Type I	83	Type III	49	Type III	125	Type III
41	Type I	23	Group IV (5)	17	Group IV ^u (3)	107	Subgroup II B (1)
57	Group IV ^u (het)	52	Group IV (6)	82	Group IV ^u (het)	131	Group IV (?)
72	Group IV ^u (het)			89	Group IV ^u (het)		
34	Group IV* (?)			94	Group IV ^u (het)		
69	Group IV* (?)			39	Group IV* (?)		
76	Group IV* (4)			65	Group IV* (?)		
2	Group IV (het)			78	Group IV* (?)		
71	Group IV (het)			99	Group IV* (?)		
				38	Group IV (5)		
				130	Group IV (6)		
RHINITIS AND PHARYNGITIS		INFLUENZA		INFLUENZA AND PNEUMONIA		PNEUMONIA	
Case number	Type	Case number	Type	Case number	Type	Case number	Type
18	Group IV* (?)	164	Type I	157	Type III and Subgroup II B (1)	86	Group IV (het)
169	Group IV (6)	144	Type II	168	Subgroup II A		
		146	Type II	161	Group IV ^u (3)		
		90	Subgroup II H				
		135	Subgroup II B (1)				
		166	Subgroup II B (1)				
		134	Group IV ^u (2)				
		158	Group IV ^u (2)				
		154	Group IV* (4)				
		159	Group IV* (?)				
		165	Group IV* (het)				
		156	Group IV (het)				
		160	Group IV (het)				
		163	Group IV (het)				

IV^u Strains which gave some agglutination with type II serum.

IV* Strains which gave some agglutination with all three type serums.

Figure in parentheses is an arbitrary symbol to note the groups encountered, based on agglutinin absorption.

(Het) Heterogenous on serum reactions.

(?) No homologous serums available, did not show relationships.

types gave a positive reaction. Avery² found differences in fermentation with mannite and salicin but these differences did not correlate with the serological results.

In spite of the above data, it seemed desirable to determine the fermentative characteristics of the strains classified by us as distinct groups on the basis of agglutinin absorption. Similar

TABLE 18

Tabular summary: Number of strains of each variety in relation to the case condition

GROUPS	NORMAL	CHRONIC RHINITIS	ACUTE RHINITIS	PHARYNGITIS	RHINITIS AND PHARYNGITIS	INFLUENZA	INFLUENZA AND PNEUMONIA	BRONCHO-PNEUMONIA	TOTALS
Type I.....	2	—	—	—	—	1	—	—	3
Type II.....	—	—	—	—	—	2	—	—	2
Type III.....	—	1	1	1	—	—	1×	—	4
Subgr. II A.....	—	—	—	—	—	—	1	—	1
Subgr. II B (1)....	—	—	—	1	—	2	1×	—	4
Subgr. II H.....	—	—	—	—	—	1	—	—	1
Group IV ^{II} (2).....	—	—	—	—	—	2	—	—	2
Group IV ^{II} (3).....	—	—	1	—	—	—	1	—	2
Group IV ^{II} (het.)..	2	—	3	—	—	—	—	—	5
Group IV* (4).....	1	—	—	—	—	1	—	—	2
Group IV* (het.)..	—	—	—	—	—	1	—	—	1
Group IV* (?).....	2	—	4	—	1	1	—	—	8
Group IV (5).....	—	1	1	—	—	—	—	—	2
Group IV (6).....	—	1	1	—	1	—	—	—	3
Group IV (het)....	2	—	—	—	—	3	—	1	6
Group IV (?).....	—	—	—	1	—	—	—	—	1
Total.....	9	3	11	3	2	14	3	1	

See table 17 for explanation of symbols.

×Types isolated from the same case.

groups have not been studied in this connection; the greater attention has been placed on the dominant fixed types. From one to six strains of an individual case were employed.

All the strains except two, promptly and vigorously, fermented inulin, lactose, raffinose and saccharose. The two strains from case 169 showed less avidity for inulin than the other strains.

² Results not published. Personal communication.

TABLE 19
Agglutination of strains from individual case with antiserum for one strain from the same case

CASE NUMBER	EXAMINATION NUMBER	DAYS ILL	ISOLATED FROM	SERUM USED	AGGLUTINATION TITRE OBTAINED WITH STRAINS NUMBERED							
					1	2	3	4	5	6	7	8
168	II	15(?)	m	168-V-1		500	500	500				
168	IV	22(?)	m	168-V-1	600	500		600				
166	I	3	m	166-II-1	200	100	200					
166	II	7	m	166-II-1	200	100						
166	III	13	m	166-II-1	200							
90	III	4	m	90-III-1	500	500	500	500				
158	I	2	m	158-I-1	400	300	400	400				
158	II	7	m	158-I-1	400	400	400	300				
17	I	1	m	17-I-1	400							
17	I	1	pl	17-I-1		400						
72	II	—*	m	72-II-4	500	600	600	600				
89	II	2	m	89-II-1	300							
89	III	?	m	89-II-1	300	300						
57	I	—	m	57-I-5	200	200	200	200	200	200		
94	I	1	m	94-I-5	300	300	300	300	300	300		
76	I	—	m	76-I-1	300	300	300	300	300	300		
154	I	1	m	154-I-1	400	400	400	400	400			
165	I	4	m	165-I-1	200		200					
38	II	$\frac{1}{2}$	m	32-II-2		400	400	400	400			
52	I	?	m	52-I-1	200	200	300	200	200			
130	I	$\frac{3}{4}$	m	130-I-1	600	600	500	600	700			
2	II	—	m	2-II-1	400	500	300	400	500			
71	I	—	pl	71-I-5	400		400		400	400		
86	I	9	pl	86-I-1	300	400	300	300	300	400	300	
156	I	2	m	156-I-1	300	200	300	300		300		
160	I	2	pl (a)	160-I-1 (a)	300	200	200	200	200	200	300	200
160	I	2	pl (b)	160-I-1 (a)	200	200	200	200	200	200	200	200
160	I	2	m	160-I-1 (a)	200	200	300	300				
163	I	2	m	163-I-1	200	200	200	200				

* Symbol — = normals.

TABLE 20
Agglutination of strains from individual case with antiserum for strains shown to be the same on agglutinin absorption

CASE NUMBER	EXAMINATION NUMBER	DAYS ILL	ISOLATED FROM	SERUM USED	AGGLUTINATION TITRE OBTAINED WITH STRAINS NUMBERED				AGGLUTINATION TITRE, SERUM STRAIN
					1	2	3	4	
135	I	$\frac{1}{2\frac{1}{2}}$	m	166-II-1	300	300	200	200	300
157	II	8	m	166-II-1	300	300	300	300	300
134	I	$\frac{1}{6}$	m	158-I-1	300	300	300	300	300
161	I	1	m	17-I-1	300	400	400		300
161	III	7	m	17-I-1	300	400			300

These positive results are not tabulated. The strains showed considerable variation however in their ability to ferment salicin and mannite.

Table 21 gives the fermentative results with salicin and mannite.

With some of the cultures two sets of tests were made at different dates. In each test, two or three kinds of media were employed for comparison; when one medium was used in duplicate, different methods were employed to determine the presence of fermentation. One medium (serum semi-solid) is common to both tests. This allowed of some comparison as to the constancy of the degree of fermentative capacity of the individual strains.

The broth employed consisted of a beef infusion base. The semi-solid medium had a veal infusion base and contained just sufficient agar to give a soft jelly consistency. Where not otherwise noted, the indicator was acid-fuchsin decolorized by sodium hydrate (Andrade indicator). A duplicate set of tests was made with serum broth and the pH point was determined after periods of incubation as noted in the table. Unheated horse serum was utilized in the serum media. Ten Broeck¹³ has called attention to the danger of false positive results when unheated serum is used in fermentation tests. Incubated blank tubes were employed, therefore, as controls to exclude this source of error, as well as known negative and positive control strains.

The results show a complete lack of any correlation between fermentative reaction and agglutinative results. There is a marked tendency for the results to vary on different media as well as upon the same medium. A few of the strains show a greater and a more constant tendency to ferment. Variable results were obtained even with individual fishings from a single case.

On the whole, therefore, the conclusion seems justified that the ability to ferment salicin is not a fully developed characteristic. The ability to ferment mannite is even less developed. The condition is comparable to the variable avidity for certain carbohydrates or alcohols shown by members of the typhoid-paratyphoid and dysentery groups. Such an example is found in

TABLE 21
*Fermentation reactions—mannite and salicin**

CASE NUMBER	EXAMINA- TION NUM- BER	STRAIN NO.	SALICIN						MANNITE					
			Tested 6/15/20			Tested 11/24 20			Tested 6/15/20			Tested 11/24/20		
			Serum semi- solid agar pH 7.8	Semi- solid agar pH 7.7	Beef- broth pH 7.6	Serum semi- solid agar pH 7.5	Serum beef- broth pH 7.3	Serum beef broth pH 7.4	Serum semi- solid agar pH 7.7	Semi- solid agar pH 7.7	Beef- broth pH 7.6	Serum semi- solid agar pH 7.5	Serum beef- broth pH 7.3	Serum beef broth pH 7.4
													2 days	7 days
166	I	1	+	++	-	+	+		-	-	-	-		
		2	+	+	+	+			-	-	-	-		
		3	+	+	++	+			-	-	-	-		
166	II	1	+	+	+	+	+	5.3	+	+	-	-	6.8	6.6
		2	-	-	-	+		6.6	+	+	-	-	6.8	6.6
107	I	1	+	+	-	+	+	5.0	+	-	-	±	-	6.0
135	I	1	+	+	-	+	+	5.2	+	+	-	-	6.7	6.35
157	II	1	+	+	-	+	-	5.0	+	+	-	-	-	6.7
158	I	1	+	+	±	+	+	5.1	+	+	-	-	6.8	6.7
		2	+	+	±	+	+	5.1	+	+	-	-	6.8	6.7
		3	+	+	-				-	-	-	-		
		4	+	±	-				-	-	-	-		
158	II	1	+	+	-				-	-	-	-		
		2	+	+	-				-	-	-	-		
		3	+	+	-				-	-	-	-		
134	I	1	-	-	-	±	+	5.2	+	+	-	-	6.8	6.7
		2	+	±	-				-	-	-	-		
		3	+	+	++	+			-	-	-	-		
		4	+	+	++	+			-	-	-	-		
17	I	1	+	+	+	+	-	5.15	+	+	-	-	6.8	6.5
		2	+	+	+	+	+	5.15	+	+	-	-	6.6	6.5
161	I	1	+	+	+	+	+	5.2	+	+	-	-	6.6	6.0
		2	+	+	-	+			+	+	-	-		
		3	+	+	-	+			+	+	-	-		
161	III	1	+	+	+	+	+		+	+	-	-		
		2	+	+	+	+	+		+	+	-	-		

[illegible]

* All the cultures fermented Inulin, Lactose, Raffinose and Saccharose vigorously and promptly. Two cultures 169-II-1 and 169-II-2 showed slightly less avidity for inulin than the others.

† The negative controls gave hydrogen ion concentration values between pH 6.4 and pH 6.7. No figure above 6.4 can be interpreted to indicate a true fermentation of the salicin or mannite.

Symbols: ++, + and \pm degree of fermentation as shown by intensity of color change.

the varying avidity of *B. cholerae* suis for dulcitol and arabinose as shown in the work of Krumwiede, Kohn and Valentine (14).

As far as can be determined, the presence or absence of fermentation of mannite or salicin by pneumococci has no classificatory significance.

CONCLUSIONS

The serological relationship of fifty-five strains was studied. These were isolated from the naso-pharynx of normal controls, from cases of rhinitis, and pharyngitis, also from influenza-like cases and influenza-like cases complicated by broncho-pneumonia. No single variety of pneumococcus was found to be dominant in the inflammatory conditions studied. This was as true for the influenza-like cases as for common colds. At most the only difference between the normal and the acute inflammatory condition was a slightly greater incidence in the latter of the different varieties classified as sub-groups of type II. There was no data, therefore, which indicate that the pneumococcus was the primary etiological agent in the contagious types of inflammation.

In twenty-five cases from which several strains were isolated from each individual all the strains from the individual case were similar with one exception. In one case two varieties were found. These findings indicate that usually one variety of pneumococcus predominates in the naso-pharynx.

Some of the serological groups as well as individual strains, have been classified as members of the sub-groups of type II by the use of control strains. Other groups and individual strains seemingly should be classified in the same manner. Some groups belong apparently in group IV. The question whether strains should or should not be classified as belonging to the sub-groups of type II is not satisfactorily determinable with the criteria available.

The fermentative results with salicin and mannite were inconsistent and the differences among strains showed no parallelism with the serological findings. Such differences have, therefore, no evident classificatory significance.

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III. RELATIONSHIP TO UPPER RESPIRATORY INFECTIONS OF STREPTOCOCCI PRODUCING A GREEN ZONE ON STANDARD BLOOD AGAR PLATES (SMITH AND BROWN'S ALPHA TYPE)

ANNA W. WILLIAMS, AARLAUG UNNEBERG, AGNES GOLDMAN AND
HELENA HUSSEY

The primary object of this study, as we have already stated, was to look for evidence of an epidemic strain among cocci of this type.

With regard to this group of bacteria, perhaps more than any other, sweeping statements have been made concerning the relationship of strains to epidemics. This is largely due to their ubiquity and the difficulty of demonstrating clear cut proofs of such relationship and also to the ease with which deductions are made from insufficient evidence.

The most important question relating to this subject about which we have little accurate knowledge, is the question of the permanency of those characteristics which are supposed to be indicative of species, of specific pathogenicity and of related characteristics which might be used in diagnosis.

We know a little of the limits of change in *vitro* but essential changes in the animal have not yet been demonstrated with controls sufficiently free from error. Indeed, until we can employ a technic that seems now to be practically impossible in its minutia, we cannot claim to have demonstrated fundamental changes occurring in microorganisms grown in either test tube or animal.

So much has been claimed for this group of organisms, especially since the "flu" pandemic, that it seems more important than ever to obtain a more definite knowledge of them. The accounts of the strains reported as probably having an etiologic relationship to influenza are so meager that we cannot agree

with a recent statement that these strains are "probably all the same organism," especially in the light of our work on colony fishings, the first report of which appears in this paper.

The most minute recent work on cultural characteristics of "green producing streptococci" has been done by Brown (1), confirming and continuing the work started by Theobald Smith and Brown (2) in 1915. The chief advance that Brown made (1919) in studying more minutely the changes on a standard blood agar plate was to describe certain definite ice-box changes in the colonies. In his very comprehensive monograph he gave an extensive comparative table of bibliography and tried to correlate his work with that of others. He adopts, with some modifications, the classification of Holman as to the sugars.

His work on colony changes on his standard blood agar plate is most interesting in that he claims that certain small but definite appearances differentiate clearly between the so-called true hemolyzers (Smith and Brown's beta type) and the green-producing streptococci (Smith and Brown's alpha type).

We are using the term "alpha" as stated by Smith and Brown in 1915, although Brown in the introduction of his 1919 monograph says that they avoided the term *Streptococcus mitior seu viridans* for the less hemolytic streptococci because many of them produced little or no green color in blood agar plates. All of our strains reported in this paper were fished as green-producing colonies from Brown's standard-blood-agar plates. We are also accepting tentatively the broader description of hemolysis that Smith and Brown recognize, namely, that of a passing of hemoglobin from the red blood cells from whatever cause and that any lightening of the medium about a colony in a standard blood agar plate is evidence of a corresponding degree of hemolysis in this general sense.

As we said, the strains reported in this paper were originally fished from colonies that appeared to be of the alpha type and in the influenza cases all apparently of one type of alpha which we labeled "minute greens." These fishings were subsequently studied with great detail on blood agar plates following exactly Brown's method as reported in his monograph.

They were also studied for morphologic and staining characteristics on chocolate agar (p. 13) and in pneumococcus broth (p. 24). They were all Gram-positive cocci growing chiefly in medium-length chains in the broth and in pairs on chocolate agar. On the latter medium the individual organisms were frequently elongated. The morphology of the organisms in a few of the isolations differed in some particulars from that of others but whether these are lasting differences we do not know. The bile test in all of them was negative. Their reactions were tested to seven carbohydrates, namely, dextrose, saccharose, mannite, lactose, salicin, raffinose and inulin. Duplicate tests were made with the addition of serum from defibrinated horse blood. The medium was made up as follows:

To standard sugar-free veal infusion (1.5 per cent agar), 1 per cent sugar was added. The sugars were made up in 5 per cent solutions and sterilized in the Arnold for one half hour on 3 successive days. The sugars were added to the finished melted nutrient agar just before it was tubed and 1 per cent standard Andrade indicator was added at same time. 15 per cent serum from defibrinated horse blood was added last.

Smith and Brown had described a sub-group of their alpha type based on colony formation which they called "complex." Brown described a sub-group based on blood change which he calls "alpha prime."

After studying some of our fishings the following tentative classification was made by us to aid in recording our results.

Colony types in standard blood-pour plates formed by Gram-positive bile-negative streptococci producing green coloration, and central area of corpuscles (Smith and Brown's alpha type)

Alpha I

Colonies—simple and small to minute.

Zone of lightening.

Before refrigeration.

Size approximately equal to colony.

Distinct green coloration—with corpuscles throughout.

After refrigeration.

Size slightly enlarged.

Disappearance of green coloration with more clearing.

After second incubation.

Size same.

Further clearing.

Second green zone irregular in appearance dependent somewhat upon degree of dilution of culture.

Alpha II

Colonies intermediate between simple and complex and less minute than I.

After end incubation they become on the whole more complex. Zone of lightening and second green zone as in Type I.

Alpha III

(Smith and Brown's complex colonies)

Colonies complex.

Zone of lightening and second green zone as in Type I.

Alpha IV

(Smith and Brown's "alpha prime?")

Colonies simple and small.

Zone of lightening.

Before refrigeration.

Size larger than colony—marked clearing.

Haziness or green zone along edges.

After refrigeration.

Size probably little or not at all enlarged.

Further clearing, sometimes complete destruction of red blood cells after second incubation.

Further clearing and destruction of red blood cells.

Note: Central area of corpuscles after refrigeration sometimes appears more distinct macroscopically from light zone.

Second green zone variable.

After studying our strains according to this method on standard plates and in comparison with some hemolytic (beta type)

strains that we obtained in this work¹ we find that we may summarize the slight differences that we obtained from those of Brown (in differentiating the types on blood agar) by the following table.

TABLE 1

General characteristics differentiating "alpha" streptococcus types according to growth in standard blood agar plates (Brown) under standard conditions

TYPE	CENTRAL ZONE OF CORPUSCLES	GREEN-ISH ZONE	CLEAR ZONE	INCREASE OF CLEAR ZONE ON REFRIG-ERATION	COMPLEX COLONIES	HEMOLYSIS IN TEST TUBE	REMARKS
According to Brown							
Alpha	+	±	±	+	- or +*	Not reported	* Rare
Alpha prime	+	-?*	+	+	-	Not reported	* Description not clear
Beta	-	-	+	-	-	+	See Brown (3)
Gamma	-	-	-	-	-	Not reported	
According to us							
Alpha	+	+	±	±	- or ±*	-	* Comparatively frequent
Alpha prime	+	±	±	+	-	- or ±*	* Comparatively rare (Mishulow)
Beta	-	- or ±†	+	- or ±*	- or ±†	+	* Very rare (Valentine and Mishulow)
Gamma	-	-	-	-	-	Not done	† Rare

In general, we may say, our description of the types agrees with that of Brown. The slight differences we have found may be listed as follows:

1. All the strains we have studied as "alpha" produce a green zone, however delicate, on standard plates.

¹ These strains were passed over to Dr. Krumwiede's group and they are being studied by Miss Valentine and Miss Mishulow who will report their results. They have given us some observation they have already made; especially on test tube hemolysis.

2. Some of our alpha strains have shown no increase in the zone of clearing after refrigeration.

3. Our alpha strains growing as "complex colonies" are rather frequent.

4. All of the strains, which we have classed as "alpha prime," have produced at least some green color within forty-eight hours in the incubator (36°C.).

5. Some of our alpha primes have produced some hemolysis in the test tube (reported to us by Mishulow).

6. An occasional definite "beta" strain has produced a green zone on the standard plate.

7. Two strains of "betas" have markedly complex colonies. (These are the strains marked "Witt"² and "Witt-like" in the report to be given by Valentine and Mishulow.)

We have come to the conclusion that the chief distinguishing characteristic between the alpha and beta type of hemolysis on Brown's standard blood agar plate is the zone of corpuscles immediately surrounding the colony, and that this characteristic may be a more constant test of the different types than tube hemolysis.

We compared the results we obtained on the carbohydrate media with those on the Brown plates. The following table shows the comparative results from individual cases. We arranged the sugar combinations in the order given by Brown.

We are giving in this table only those groups of sugar reactions into which the great majority of our strains fell. The few fishings that fell into other sugar groupings are included in the next table.

We see from table 2 at a glance that there is no consistent relation between types on standard blood agar plates and sugar reactions. Heterogeneity of sugar types found in colony fishings of "green streptococci" from individual cases of "common colds" has been reported by Krumwiede and Valentine (4). It is true that markedly more cultures from the influenza cases than from the other two groups of cases fall into the ignavus

² Two interesting organisms were obtained from the "Witt" case, with which different groups in our laboratory have worked. One is a hemoglobophilic bacillus (see Dr. Povitzky's paper) and the other is a hemolytic streptococcus.

TABLE 3

Tabulation of alpha streptococci according to Brown's scheme of sugar reactions

MAN- NITE	LAC- TOSE	SALI- CIN	GROUPS																
-	+	I	<i>S. mitis</i>		F	30	I	F	42	I	F	1	I	F	11	I			
			F	5	II	F	7	II	F	1	II	F	2	II					
			F	10	III	F	5	III	F	1	II-III	F	1	III					
			F	1	IV	F	20	IV				F	1	I-III					
			C	7	I	C	29	I				C	1	I					
			C	1	II	C	5	II				C	3	II					
			C	2	IV	C	1	IV											
			N	8	I	C	5	I-IV											
			N	1	III	N	7	I											
			N	1	IV														
			<i>S. salivarius</i>		F	49	I	F	26	I									
			F	5	II	F	8	II											
			F	5	III	F	6	III											
			F	6	IV	F	18	IV											
			F	1	I-IV	C	15	I											
			C	5	I	C	4	IV											
			C	2	IV	N	6	I											
			N	4	I	N	1	II											
			N			N	2	III											
			<i>S. equinus</i>		F	3	I	F	2	I	C	3	I	F	2	II			
			F	1	II						N	2	I-II						
	F	1	IV																
	N	2	I																
	N	2	II																
	N	2	IV																
	<i>S. ignavus</i>		F	12	I	F	9	I											
	F	6	II	F	5	II													
	F	7	IV	F	1	III													
	C	1	II	F	6	IV													
	C			C	1	I													
	Subgroups				1		2		3		4								
Raffinose				-		+		-		+									
Inulin				-						+									
Saccharose								+											

F = influenza.

C = colds.

N = normal.

Arabic numbers = number of fishings.

Roman numbers = subdivisions of alpha type on blood agar plates.

columns, but these are divided in the two columns and in each column several varieties of blood-agar-plate colonies are recorded. Furthermore, the larger number of cases and the greater number of isolations made among the influenzas may account for this predominance in the ignavus columns. The isolations from each case are irregular in regard to the above tests and most irregular in the influenza cases. It is evident then that, as far as these cultural reactions are concerned, a multiplicity of strains exists.

Table 3 gives, according to Brown's sugar reaction scheme, an arrangement of the blood plate types from the three different groups of cases as a whole. We needed only to record the first four groups of Brown and their first four sub-groups; that is, the upper left hand corner of his scheme, since we had no cultures among our isolations that fell into the other groups.

Here again we see that no one combination predominates significantly over the others in any clinical case group.

In our work on direct agglutination and the absorption of agglutinins, which will be reported in a later paper, we have been able thus far to show that a serum produced by a culture having certain blood plate and sugar fermentation characteristics may not react with all of the fishings from that case showing the same cultural characteristics.

The only general statement we can make so far from this study is that in a series of influenza cases, cultures isolated from colonies of an apparently similar type belonging to the "alpha streptococci" and appearing as dominants, show a multiplicity of strains as indicated by the reactions with carbohydrates and with a standard blood-agar medium.

Although these cultural reactions may not be as fixed as certain immunological reactions, it remains to be demonstrated that these cultural reactions can change in their passage from individual to individual throughout a single epidemic.

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IV. FURTHER STUDIES ON GROUPING OF INFLUENZA BACILLI WITH SPECIAL REFERENCE TO PER- MANENCE OF TYPE IN THE CARRIER

OLGA R. POVITZKY AND HELEN T. DENNY

ASSISTED BY DOROTHY J. PROVOST AND ANNA C. LAMONT

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The first large series of experiments on the relationship of the influenza bacillus to epidemic influenza was carried out at the Research Laboratory under the direction of Park, Williams and Krumwiede (1). The serological work on grouping was done by Valentine and Cooper (2). They worked with 171 strains isolated by Williams from different sources during the pandemic of 1918. These strains were tested by the agglutinin absorption method with 25 antisera. The only grouping of strains into types, that could be obtained by this method was as follows: Strains from two individuals among a group of marines infected with influenza were found to be identical. Strains from two children at a children's home also gave identical reactions. As no other definite relationships could be established among the large number of strains tested, the conclusion seemed justified that under the term of *Bacillus influenzae*, just as with the term pneumococcus, we are dealing with a group of heterogeneous organisms. In the case of the influenza bacillus, there is even less suggestion of any dominant types. The existence of a multiplicity of types in cases attacked at the same time and in the same locality, was advanced as evidence against *B. influenzae* being the primary etiological agent in epidemic influenza.

Huntoon and Hannum (3) state that there were no strains among their cultures which did not show relationship, either directly or indirectly through absorption tests. The titer of their serums was rather low for identifying strains by absorption tests. It is extremely probable that with monovalent serums of higher

titer many more differences would have been discovered. Moreover, some of their tables show distinct type differences by absorption tests.

Small and Dickson (4) studied ten strains and obtained four type groups from nine strains by subjecting them to agglutinin absorption tests.

Rivers (5) from his investigations thinks that the influenza bacillus can be divided into groups according to their biological characteristics. Thus, he found one definite group of ten strains all of which were similar culturally, all formed indol, all reduced nitrates to nitrites and made blood broth milk slightly acid within forty-eight hours. Besides these he found some strains which produced hemolysis.

In spite of the general agreement in the results of the different investigations, it was thought wise to obtain a large number of new strains and separate them into their immunological types by direct agglutination and agglutinin absorption tests. We utilized about 100 old strains most of which had been isolated by Williams during the pandemic of 1918 and 90 new strains. These last had been isolated by one of us from a series of cases studied by Williams in connection with the recent outbreak of influenza. We had 30 sera, some of which were prepared from the strains of the pandemic, but most from the strains isolated previous to and during the recent outbreak. We tested all our old and new strains with these 30 sera at different times and we state at once that our results are in close agreement with those obtained by Valentine and Cooper. In cases of influenzal meningitis, we were so fortunate as to have discovered a dominant type of *B. influenzae*. We also added three more strains to the Leuchner group of Valentine and Cooper, thus identifying four strains in the meningitis group and five strains in the Leuchner group (tables 1 and 2). A great many other strains were agglutinated to a high titer by heterologous serums but by absorption tests were proven to be alike only in their abundant group agglutinins (table 3).

As to the cultures isolated previous to and during the recent epidemic, none except a few contact cases were found to be iden-

tical in type, though a great many strains seemed to be closely related as shown by the absorption of a certain amount of agglutinins. We shall, however, speak about these strains from the recent outbreak in more detail later.

In all, we had seven influenzal meningitis cultures all of which, except one, were isolated in our laboratory (Meningitis Division); of these, two had been isolated a few years before the pandemic, three during the pandemic and one since. One was given to us by Wollstein of The Rockefeller Institute. We developed antisera for two of the influenzal meningitis cultures, namely 6741 and 6746. Four of the seven influenzal meningitis cultures were agglutinated by the first serum (6741) and absorbed the agglutinins. None of the remaining three was agglutinated by or absorbed the second serum except the homologous strain. Culture 6746 is, therefore, a distinct type. The remaining two cultures were not identical with either of the above types. As to the Leuchner type, three cultures, namely Leuchner, Myers, and Bernstein were isolated from these three children in an institution. Grahs, an autopsy strain and Robenson a case from the navy, comprise the remaining two of the group of five. These two persons had no intercourse with each other or with the three children.

Before coming to the discussion of the recent epidemic, we must say a word about the difficulties encountered when working on agglutination tests with *Bacillus influenzae*. The difficulty Wollstein (6) emphasizes, namely, that this organism clumps spontaneously, was with our methods negligible. Ever since the workers in this laboratory began to grow this organism on the chocolate medium and to scrape the growth off in salt solution, instead of washing it off, a homogeneous suspension is the rule. The greatest difficulty in the past has been the capriciousness and unreliability of the influenza bacillus as to its agglutinability. Some time ago, we noticed that the cultures used for animal inoculation and therefore transplanted each day, could be relied upon more surely for their agglutinability than the same cultures kept in stock and transplanted only once a week. In order, therefore, to restore to cultures their lost agglutinability we have transplanted them every day for at least a week before using

them in a test. In the case of convalescent carriers, we noticed also that when we had a serum ready, the cultures if freshly passed through animals responded much more promptly to agglutination than they had done before or did a little later on. The loss in agglutinability was noticeable within a week's time after isolation. One of our cultures, namely Witt (isolated by Williams from a case of pleurisy following influenza and used by Cecil (12) for his experiments) when tested, failed altogether to agglutinate, but the same culture when passed through the monkey agglutinated beautifully if transplanted every day for one week before doing the tests. As to the serum, if it is of a good titer and agglutinates the culture in large clumps, it keeps for long periods without appreciable deterioration in the ice box. We have used such a serum over a period of two years without noting any considerable diminution of its titer.

Our technique for agglutination and absorption tests was the same as that employed by Valentine and Cooper except in some minor details. Cultures grown on chocolate medium for twenty-four hours were scraped off in very small amounts of salt solution and well shaken; after an even suspension was made it was diluted with 0.85 per cent salt solution to the standard opalescence. The tubes received suspension and decreasing amounts of a serum diluted 1:25 so as to make final dilutions of from 1:50 to 1:2500, etc.

The tests were put in the water bath at 45°C. for two hours. Readings were made after two hours and again the next morning.

For absorption tests, twenty-four to forty-eight hour growths on chocolate medium were obtained by using 7 to 9 potato tubes for each culture to be tested. The growth was scraped off in a small amount of salt solution, centrifugalized and the packed bacteria treated according to the following formula:

If the titer of the serum was between 1:700 and 1:800, the packed cells were multiplied by 10 to obtain a figure for the total volume of packed cells and diluted serum; if above this titer, by 5. From this, half the volume of the cells was deducted to allow for the remaining water (after centrifugalizing) and the result divided by 10 or 15 depending on whether the primary dilution of the serum was to be 1:10 or 1:15. The estimated amount of undiluted serum was added to the packed cells and enough salt solution to bring the whole to the total volume so estimated above.

To illustrate, suppose we have packed cells to the amount of 0.2 cc. and the titer of the serum is 1:800. The formula in this instance will be: $0.2 \times 10 = 2$ cc. — $0.1 = 1.9:10 = 0.19$.

The amount of serum to make a primary 1:10 dilution is 0.19 cc. and salt is added to make a volume of 2 cc.

For our work, we used centrifuge tubes graduated in 0.1 cc. After each mixture of serum and organisms had been stirred thoroughly, it was put in the water bath at 45°C. for three hours and shaken from time to time to keep the organisms in even contact with the serum. It was then put in the ice box over night.

Agglutination tests were set up the next day with the centrifugalized supernatant fluids against the strains used for absorption and the serum strain. Controls for the potency of the unabsorbed serum with the same strains and the usual controls for the plain suspension, were always made.

INVESTIGATION OF THE STRAINS FROM INFLUENZA-LIKE CASES IN RETURN OUTBREAK OF THE WINTER, 1920

The object of our studies was twofold: First, to test again the possibility of the constant presence of a dominant type of influenza bacillus which we had failed to find during the pandemic of 1918. If such a type were found, it might be considered the cause of the original pandemic as well as the return outbreak.

Second, to determine the persistence of type characteristics in the strains found in convalescent cases and in normal carriers of the influenza bacillus. If type characteristics were stable, then the finding of many different types in the epidemic cases occurring at about the same time would militate still more strongly against the etiological significance of the influenza bacillus.

With these objects in view, we fished from each case at least 10 colonies and from selected cases as many as 30 of both small and large influenza-like colonies. Each colony from an Avery medium plate was transplanted on chocolate medium (reaction pH 7.2 to 7.5). Each culture from each separate colony was tested and retested as to its hemoglobinophilic properties by trying it on plain and ascitic agar and studied for its morphology. We utilized

the morphology of the hemoglobinophilic organisms to separate the atypical from the typical hemoglobinophilic bacilli.

Atypical hemoglobinophilic bacilli. These are bizarre, long and curved bacilli, often growing out in threads. They seem to correspond to the organism which Pritchett and Stillman (7) name "the undescribed bacillus X." We found these organisms as the sole hemoglobinophilic organism in 18 cases (some normals and some colds) previous to and during the recent epidemic and in 5 cases coexistent with typical influenza bacilli. Therefore, we felt it worth while to study them more in detail. We found that on the chocolate medium they do not grow as profusely as the typical forms and that they produce a mucoid growth. They all produce hemolysis, though in varying degree, in tubes with rabbits' washed red cells and in plates with defibrinated horse blood. The hemolytic colonies on superficial examination might be mistaken for hemolytic streptococcus colonies. These organisms do not appreciably ferment glucose as the majority give no reaction and a few show but a trace. They do not produce indol. By means of two sera produced by immunizing rabbits with two different strains, we were able by absorption tests to separate two groups of two and three each; the rest do not belong to either of these two groups and may or may not form many independent groups. Direct agglutination tests are not satisfactory as most of the bacilli clump spontaneously.

Typical hemoglobinophilic bacilli. The morphology of these was such as we usually associate with a typical influenza bacillus; a small slender rod often coccoid in form, later becoming more irregular and often growing out in threads. It grows profusely on the chocolate medium, between pH 7.2 to 7.5 or 0.2 to 0.3 acid to phenolphthalein (neutral to phenolphthalein before autoclaving).

In order to study the agglutinating characteristics of our separate colonies, we produced over thirty different sera from selected cases.

Rabbits chosen for inoculation weighed between 2000 and 2500 grams. Inoculations were given on three successive days if the condition of the animal allowed and three days rest followed. This proc-

ess was repeated as often as necessary. An emulsion in saline of 1:40 to 1:10 of the growth on one test tube slant of chocolate agar was used for the initial injection and the amount gradually increased to the growth on two slants. The rabbits on the same culture were found to differ greatly in their resistance and power to form agglutinins. The average time necessary to obtain a serum giving complete agglutination in 1:2500 dilution was seven weeks and the average number of injections was 17.

TABLE I
Influenzal meningitis group of cultures

DIRECT AGGLUTINATION								AGGLUTINATION AFTER ABSORPTION														
Serum	Culture	100	200	400	600	800	1200	Serum	Culture for absorption	1:0	200	400	600	800	1:00	Serum strain	50	100	200	400	600	1000
6741	6741	+	+	+	+	+	+	6741	6741	-	-	-	-	-	-	6741	-	-	-	-	-	-
	B.I.W.	+	+	+	+	+	+		B.I.W.	-	-	-	-	-	-	6741	-	-	-	-	-	-
	Z.	+	+	+	+	+	+		Z.	-	-	-	-	-	-	6741	-	-	-	-	-	-
	747	+	+	+	+	+	+		747	-	-	-	-	-	-	6741	-	-	-	-	-	-
	6746	±	-	-	-	-	-		6746	-	-	-	-	-	-	6741	+	+	+	+	+	+
	6745	±	-	-	-	-	-		6745	-	-	-	-	-	-	6741	+	+	+	+	+	+
6746	6827	±	-	-	-	-	-	6741	6827	-	-	-	-	-	-	6741	+	+	+	+	+	+
	6746	+	+	+	+	+	+															
	6741	±	-	-	-	-	-															
	Z.	±	-	-	-	-	-															
	6745	±	-	-	-	-	-															
	6827	±	-	-	-	-	-															
	B.I.W.	±	-	-	-	-	-															

Among our most interesting studies of the recent epidemic were those made on a family of five, a mother and four children. They were taken ill with influenza at the same time and were all brought to the hospital. From 10 to 20 colonies were fished from plate cultures of each member of the family. The interesting fact was brought out that the colonies obtained from each member of the family were not all identical with each other; the individuals had probably been carriers of two or more distinct types before the new infection (see table 4). Homologous serums were prepared with organisms from two members of this family, starting with one colony from an individual in each case. These sera were tested with a number of strains from each case (table 4).

TABLE 2
Leuchner type

DIRECT AGGLUTINATION			AGGLUTINATION AFTER ABSORPTION AND INDOL FORMATION													
Serum	Culture						Cultures for absorption		Serum strain						Indol	
	100	200	400	600	800	1200	Leuchner	Myers	100	200	400	600	1000			
Leuchner	+	+	+	+	+	+	Leuchner	—	—	—	—	—	—	—	—	
	+	+	+	+	+	+	Myers	—	—	—	—	—	—	—	—	
	+	+	+	+	+	+	Bernstein	—	—	—	—	—	—	—	—	
	+	+	+	+	+	+	Robenson	—	—	—	—	—	—	—	—	
	+	+	+	+	+	+	Grahs	—	—	—	—	—	—	—	—	

TABLE 3

Showing high direct agglutination by monovalent serums for heterologous cultures but incomplete absorption of agglutinins by them for the serum strain

DIRECT AGGLUTINATION							AGGLUTINATION AFTER ABSORPTION																
Serum	Culture	100	200	400	600	800	1200	Serum	Absorption culture	50	100	200	400	600	1000	Serum strain	0	001	005	009	0001		
57	57	+	+	+	+	+	+	57	57	-	-	-	-	-	-	57	+	+	+	+	+	+	
	Meisner	+	+	+	+	+	+		Meisner	-	-	-	-	-	-		+	+	+	+	+	+	+
	J5	+	+	+	+	+	+		J5	-	-	-	-	-	-		+	+	+	+	+	+	+
	Baron	+	+	+	+	+	+		Baron	-	-	-	-	-	-		+	+	+	+	+	+	+
Margolin	Pambroke	+	+	+	+	+	-	Margolin	Pambroke	-	-	-	-	-	-	Margolin	+	+	+	+	+	+	
	Margolin	+	+	+	+	+	+		Margolin	-	-	-	-	-	-		+	+	+	+	+	+	+
	Masates	+	+	+	+	+	+		Masates	-	-	-	-	-	-		+	+	+	+	+	+	+
	6746	+	+	+	+	+	+		6746	-	-	-	-	-	-		+	+	+	+	+	+	+
6746	76	+	+	+	+	+	+	6746	76	-	-	-	-	-	-	6746	+	+	+	+	+	+	
	6741	+	+	+	+	+	+		6741	-	-	-	-	-	-		+	+	+	+	+	+	+
	42	+	+	+	+	+	+		42	-	-	-	-	-	-		+	+	+	+	+	+	+
	67	+	+	+	+	+	+		67	-	-	-	-	-	-		+	+	+	+	+	+	+
6741	Friedman	+	+	+	+	+	+	6741	Friedman	-	-	-	-	-	-	6741	+	+	+	+	+	+	
	Larsen	+	+	+	+	+	+		Larsen	-	-	-	-	-	-		+	+	+	+	+	+	+
	Godfrey	+	+	+	+	+	+		Godfrey	-	-	-	-	-	-		+	+	+	+	+	+	+
	Joseph	+	+	+	+	+	+		Joseph	-	-	-	-	-	-		+	+	+	+	+	+	+
6741	M22	+	+	+	+	+	+	6741	M22	-	-	-	-	-	-	6741	+	+	+	+	+	+	
		+	+	+	+	+	+			-	-	-	-	-	-		+	+	+	+	+	+	+

X = trace.

TABLE 4

AGGLUTINATION BEFORE ABSORPTION						AGGLUTINATION AFTER ABSORPTION												
Serum	Culture	100	200	400	600	800	1200	Absorbed serum	Culture	0.2	0.01	Serum strain	50	100	200	400	600	1000
142 ₁ ¹	142 ₁ ¹	+	+	+	+	+	+	142 ₁ ¹	142 ₁ ¹	—	—	142 ₁ ¹	+	—	—	—	—	—
	142 ₁ ¹	+	+	+	+	+	+	142 ₁ ¹	142 ₁ ¹	—	—	142 ₁ ¹	+	—	—	—	—	—
	142 ₁ ¹	+	+	+	+	+	+	142 ₁ ¹	142 ₁ ¹	—	—	142 ₁ ¹	+	—	—	—	—	—
	142 ₁ ¹	+	+	+	+	+	+	142 ₁ ¹	142 ₁ ¹	—	—	142 ₁ ¹	+	—	—	—	—	—
	142 ₁ ¹	+	+	+	+	+	+	142 ₁ ¹	142 ₁ ¹	—	—	142 ₁ ¹	+	—	—	—	—	—
	142 ₁ ¹	+	+	+	+	+	+	142 ₁ ¹	142 ₁ ¹	—	—	142 ₁ ¹	+	—	—	—	—	—
	142 ₁ ¹	+	+	+	+	+	+	142 ₁ ¹	142 ₁ ¹	—	—	142 ₁ ¹	+	—	—	—	—	—
	142 ₁ ¹	+	+	+	+	+	+	142 ₁ ¹	142 ₁ ¹	—	—	142 ₁ ¹	+	—	—	—	—	—
	142 ₁ ¹	+	+	+	+	+	+	142 ₁ ¹	142 ₁ ¹	—	—	142 ₁ ¹	+	—	—	—	—	—
	142 ₁ ¹	+	+	+	+	+	+	+	142 ₁ ¹	142 ₁ ¹	—	—	142 ₁ ¹	+	—	—	—	—
145 ₂ ¹	145 ₂ ¹	+	+	+	+	+	+	145 ₂ ¹	145 ₂ ¹	—	—	145 ₂ ¹	+	—	—	—	—	—
	145 ₂ ¹	+	+	+	+	+	+	145 ₂ ¹	145 ₂ ¹	—	—	145 ₂ ¹	+	—	—	—	—	—
	145 ₂ ¹	+	+	+	+	+	+	145 ₂ ¹	145 ₂ ¹	—	—	145 ₂ ¹	+	—	—	—	—	—
	145 ₂ ¹	+	+	+	+	+	+	145 ₂ ¹	145 ₂ ¹	—	—	145 ₂ ¹	+	—	—	—	—	—
	145 ₂ ¹	+	+	+	+	+	+	145 ₂ ¹	145 ₂ ¹	—	—	145 ₂ ¹	+	—	—	—	—	—
	145 ₂ ¹	+	+	+	+	+	+	145 ₂ ¹	145 ₂ ¹	—	—	145 ₂ ¹	+	—	—	—	—	—
	145 ₂ ¹	+	+	+	+	+	+	145 ₂ ¹	145 ₂ ¹	—	—	145 ₂ ¹	+	—	—	—	—	—
	145 ₂ ¹	+	+	+	+	+	+	145 ₂ ¹	145 ₂ ¹	—	—	145 ₂ ¹	+	—	—	—	—	—
	145 ₂ ¹	+	+	+	+	+	+	145 ₂ ¹	145 ₂ ¹	—	—	145 ₂ ¹	+	—	—	—	—	—
	145 ₂ ¹	+	+	+	+	+	+	+	145 ₂ ¹	145 ₂ ¹	—	—	145 ₂ ¹	+	—	—	—	—
146 ₄ ¹	146 ₄ ¹	+	+	+	+	+	+	146 ₄ ¹	146 ₄ ¹	—	—	146 ₄ ¹	+	—	—	—	—	—
	146 ₄ ¹	+	+	+	+	+	+	146 ₄ ¹	146 ₄ ¹	—	—	146 ₄ ¹	+	—	—	—	—	—
	146 ₄ ¹	+	+	+	+	+	+	146 ₄ ¹	146 ₄ ¹	—	—	146 ₄ ¹	+	—	—	—	—	—
	146 ₄ ¹	+	+	+	+	+	+	146 ₄ ¹	146 ₄ ¹	—	—	146 ₄ ¹	+	—	—	—	—	—
	146 ₄ ¹	+	+	+	+	+	+	146 ₄ ¹	146 ₄ ¹	—	—	146 ₄ ¹	+	—	—	—	—	—
	146 ₄ ¹	+	+	+	+	+	+	146 ₄ ¹	146 ₄ ¹	—	—	146 ₄ ¹	+	—	—	—	—	—
	146 ₄ ¹	+	+	+	+	+	+	146 ₄ ¹	146 ₄ ¹	—	—	146 ₄ ¹	+	—	—	—	—	—
	146 ₄ ¹	+	+	+	+	+	+	146 ₄ ¹	146 ₄ ¹	—	—	146 ₄ ¹	+	—	—	—	—	—
	146 ₄ ¹	+	+	+	+	+	+	146 ₄ ¹	146 ₄ ¹	—	—	146 ₄ ¹	+	—	—	—	—	—
	146 ₄ ¹	+	+	+	+	+	+	+	146 ₄ ¹	146 ₄ ¹	—	—	146 ₄ ¹	+	—	—	—	—
Mother's cultures absorbed by her serum strain								142 ₁ ¹	142 ₁ ¹	—	—	142 ₁ ¹	+	—	—	—	—	
Alfred's cultures absorbed by mother's majority strain serum								142 ₁ ¹	142 ₁ ¹	—	—	142 ₁ ¹	+	—	—	—	—	
Alma's culture absorbed by mother's majority strain serum								143 ²	143 ²	—	—	143 ²	+	—	—	—	—	
Theodore's culture absorbed by mother's majority strain serum								144 ₁₀ ^L	144 ₁₀ ^L	—	—	144 ₁₀ ^L	+	—	—	—	—	
Margaret's cultures absorbed by mother's majority strain serum								146 ₁₀ ^L	146 ₁₀ ^L	—	—	146 ₁₀ ^L	+	—	—	—	—	
Alfred's cultures absorbed by Alfred's serum strain								145 ₂ ¹	145 ₂ ¹	—	—	145 ₂ ¹	+	—	—	—	—	
With mother's majority colony								142 ₁ ¹	142 ₁ ¹	—	—	142 ₁ ¹	+	—	—	—	—	
Alma's								143 ²	143 ²	—	—	143 ²	+	—	—	—	—	
Theodore's								144 ₁₀ ^L	144 ₁₀ ^L	—	—	144 ₁₀ ^L	+	—	—	—	—	
Margaret's								146 ₄ ^L	146 ₄ ^L	—	—	146 ₄ ^L	+	—	—	—	—	

* Spontaneous agglutination.

X = trace.

To sum up the family table; all except two of the colonies fished from the mother's plates were identical with the serum strain. One of Alfred's colonies was identical with mother's majority strain; the other three were not. One of Theodore's and one of Margaret's colonies were identical with mother's majority strain. All the other strains were different. Four of Alfred's colonies were identical with Alfred's serum strain; three belonged to the atypical hemoglobinophilic bacilli producing hemolysis. One colony was identical with mother's majority strain; two colonies did not belong to any of the groups. One of Alfred's colonies and one of Margaret's colonies, tested by absorption, were identical with Alfred's serum strain.

Outside of this family, in other cases for which we had sera, many colonies were fished and usually showed the same type of organisms. Thus we fished from case 126, 30 colonies, large and small. In case 76, cultures were taken twice with a week's interval between; 10 colonies were fished each time. So also three sets of cultures were taken at week intervals from case 39; again 10 colonies were fished each time. All colonies in each case proved to be identical by direct agglutination and agglutinin absorption with their homologous serums. The same was true of cases 101 and 132.

PERMANENCE OF AGGLUTINATION CHARACTERISTICS DURING DISEASE AND THROUGHOUT CONVALESCENCE

Carriers. Since the pandemic of 1918, Pritchett and Stillman (8) also Winchell and Stillman (9) found influenza bacilli in 45 per cent of the throats of convalescents and normal individuals. The duration of the carrier state lasted at times from four to five months. Opie, Friedman and Blake (10) found *Bacillus influenzae* in over 35 per cent of healthy throats in Camp Funston. They had no way, however, of telling whether the organism remained of the same type or was replaced by another type. The same question is brought up by Bloomfield (11). In 1918, immediately after the epidemic, we examined convalescent carriers with homologous serums which had been prepared at the time of infection. In one of these cases (still a carrier at the end of the second month) the type remained the same.

From a second case, 50 colonies were fished, 49 of which proved to be identical with the serum strain. One colony was not identical. Two to three weeks later, 10 more colonies were fished from the same throat and none of these were identical with the former 49 colonies, but all were identical with the one strain which was different from the 49. In one case at the Hebrew Orphan Asylum, a culture taken one year later has proved by agglutination and agglutinin absorption tests to be of the same type as that of the year before. But in another case at the same asylum, the culture was of a type different from that of the year before.

In a few of our new cases just before the winter outbreak (for example, case 39 in which cultures were taken once a week for three weeks) the type remained the same by serological tests. The same was true of case 76; two subsequent fishings, one week apart proved to be the same, as well as the organism recovered from the mouse. The cases of accidental infection reported in paper 5 of these studies, are of importance not only because of being due to cultures but also because of the proof of the stability of type during the infection.

A most interesting illustration of this is a case of Cecil (12) in his experiments on the infectivity of *Bacillus influenzae*. A volunteer, Loselle was infected with the exudate from the monkey which had been inoculated with one of our pleurisy cultures (Witt). For almost three months he remained a carrier of that same type of organism (see table 5). On the other hand, another of Cecil's cases, Burns, who was a carrier before he was inoculated with culture Witt which had been recovered from the above case, showed on the third day the following among seven colonies: two colonies composed of strains identical with Witt and one unlike Witt; also four colonies which were mixtures of Witt and other types. One week later, no colonies of the Witt type were found. Another of Cecil's cases, Small, inoculated with the Witt strain, showed the culture to be still of the same type after the fifth day.

Crane (Cecil case) was tested culturally at intervals after inoculation with the Witt strain. This strain was found to be present at the end of one hour and also on the third day; but one week

TABLE 5

RABBIT	DIRECT AGGLUTINATION		AGGLUTINATION AFTER ABSORPTION															
	Serum	Culture	Serum	Culture for absorption	50	100	200	400	600	1000	Serum strain	50	100	200	400	600	1000	
791	Witt	M. W.	Witt	L. W.	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	
	Witt	L. W.*	Witt	Crane ¹	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	
	4/13	W. Crane ¹	Witt	Crane ²	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	
	4/14	W. Crane ²	Witt	Crane ³	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	
	4/21†	W. Crane ³	Witt	Burns ^b	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	
	Before infection	Burns ^a		Burns ³	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	
	1 hour after infection	W. B. ^b		Burns ³	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	
	3 days after infection	B ₁ ¹		Burns ³	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	
		B ₃ ³		Burns ³	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	
		B ₃ ³		Burns ³	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	
		B ₄ ³		Burns ³	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	
		B ₂ ²		Burns ³	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	
		B ₆ ³		Burns ³	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	
		B ₇ ³		Burns ³	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	
	1 week after	Burns ^c		Burns ^c	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	
	4/22	Smile ¹		Burns ^c	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	
	4/23	Smile ²		Burns ^c	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	
	4/27	Smile ³		Burns ^c	+	+	+	+	+	+	M. Witt	+	+	+	+	+	+	

* Nine cultures from one throat in course of three months.

† Relapse.

X = trace.

TABLE 6
Indol and glucose reactions

GROUP	CULTURE	INDOL	GLUCOSE 48 HOURS
Meningitis.....	6741*	+	+
	747	+	+
	B. I. W.	-	+
	Z.	+	+
	6746	-	+
	6745	+	+
	6827†	+	+
Leuchner.....	Leuchner†	-	±
	Myers	-	+
	Bernstein	-	-
	Robenson	-	±
	Grahs	-	±
Family group‡.....	142 $\frac{1}{2}$	+	±
	142 $\frac{2}{10}$	+	±
	142 $\frac{2}{L_2}$	+	+
	142 $\frac{5}{L_7}$	+	+
	145 $\frac{L}{2}$	+	+
	144 $\frac{L}{10}$	+	+
	143 $\frac{2}{2}$	+	±
	145 $\frac{L}{9}$	+	+
	146 $\frac{L}{4}$	+	+
	146 $\frac{L}{10}$	+	±
	145 $\frac{L}{6}$	- (atypical)	×
	145 $\frac{L}{1}$	- (atypical)	×
Miscellaneous cultures.....	12 $\frac{1}{1}$	±	+
	12 $\frac{2}{2}$	-	±
	101 $\frac{L}{4}$	++	+
	132 $\frac{1}{1}$	+	contam.
	140	±	±
	170	+	+
	175	+	+
	51	+	±
	56	+	+
	Masates	-	+
	Lee	±	±
	156	-	±
	106	++	×
	Benson	×	-
	Zweck	×	-
Atypical hemoglobinophilic cultures.....	Barley	-	-
	37T4	-	×
	33	-	×
	11 $\frac{3}{3}$	-	±
	87	-	×
	42	-	±

* First four meningitis cultures alike serologically.

† All five cultures alike serologically.

‡ Serologically the family cultures are not all alike but all form indol except the atypical cultures.

later, after a relapse, the culture taken was not of the Witt type when tested by direct agglutination and by agglutinin absorption. The later disease, if due at all to influenza bacilli, was probably due to a new infection.

Cultural types by indol tests. Jordan (13) found that 10 out of 13 strains of *Bacillus influenzae* formed indol. We were able to corroborate his tests by using the paradimethylamido benzaldehyde method that he employed. Out of 42 typical influenza strains, 28 gave indol and 2 more gave traces of indol. None of the atypical hemoglobinophilic bacilli formed indol. We selected our cultures from among different groups in order to see how far the serological tests would correspond with the indol tests and glucose fermentation tests (see table 6; meningitis group, Leuchner group, family group).

In the Leuchner group no cultures formed indol. In the meningitis group, three formed indol and one did not although they all were identical serologically; in the family table, all the cultures tested showed indol except those which belonged to the atypical hemoglobinophilic bacilli.

SUMMARY

1. Four out of seven influenzal meningitis strains isolated years apart, were proved by agglutinin absorption tests to be of one type; the remaining three were of different types. From the respiratory cases, five strains from different individuals were obtained which were of the same type. No other groups of more than two members were obtained in agglutination tests with any cultures. This was true of both the original pandemic and also of the new outbreak strains.

2. Atypical hemoglobinophilic bacilli were found in 18 cases (normal individuals and cases of cold) and in five cases these were found in coexistence with typical influenza bacilli. These atypical organisms produced hemolysis, did not form indol and did not ferment glucose.

3. About 75 per cent of typical influenza bacilli form indol; from 50 to 60 per cent ferment glucose somewhat irregularly and not energetically.

4. The indol reactions did not correspond fully among the meningitis group of four serologically identical strains. Of these, three formed indol—one did not. In the family group, though the strains were not all identical serologically, all except the atypical hemoglobinophilic colonies formed indol. In general, it was found that strains having the same immunological characteristics had the same cultural reactions, but there were some exceptions.

5. Many colonies were fished from each of a number of selected cases and tested with homologous and heterologous serums. In a few cases as many as 20 or 30 colonies from each case were tested with the homologous serum prepared from one of these colonies. In most cases the fishings of influenza bacilli proved to be of one type; at least one type greatly predominated. But in a minority of instances, the colonies from the same case were not of the same type as proved by agglutination absorption tests.

6. In carriers or where the infection was mild, more than one type variety of the influenza bacillus was apt to be found in the same case.

7. In several convalescent carriers, tests were made during periods of from several weeks to three months. These resulted in finding in all but one, the original type of the influenza bacillus. In one case, tests made one year apart proved the original type present.

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V. ACCIDENTAL INOCULATION OF INFLUENZA BACILLI ON THE MUCOUS MEMBRANES OF HEALTHY PERSONS WITH DEVELOPMENT OF INFECTION IN AT LEAST ONE. PERSISTENCE OF TYPE CHARACTERISTICS OF THE BACILLI

WILLIAM H. PARK AND GEORGIA COOPER

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The three accidental inoculations of influenza bacilli (eighteen hour cultures on chocolate agar), are of interest from the fact that infection developed in one case on the fourth day; possibly in one case on the fourteenth day and not at all in the third case. All three persons, however, became persistent carriers of the bacilli and in all cases the strains held their agglutination characteristics unchanged during the period of the tests. The details of three accidental inoculations are as follows:

Case I. It is difficult to judge whether this case was really one of infection from inoculation with the Pfeiffer bacillus or not because of the long period of incubation. Dr. H. accidentally inoculated her throat with the Lee culture, an autopsy strain from an epidemic case, while expelling a culture suspension from a syringe. The culture was an eighteen-hour growth on "chocolate blood" mixed with human serum. It happens that some time before the accident a culture from Dr. H. had been made and gave no influenza bacilli. No symptoms developed for two weeks when she developed a temperature of 101, prostration and general pains. She was confined to bed. On her return, plate cultures made from the nasopharynx showed abundant influenza bacilli.

Ten colonies were fished from a pharyngeal culture.

All isolations were alike and identical in type with the accident strain. The direct agglutinations are given in table 1. Two of the strains were further investigated by agglutinin absorption tests with the Lee serum (table 2). The identity of the Lee cultures and the H. strains was

confirmed. Sixty-eight days after the accident 11 more strains were isolated from a nasopharyngeal culture. These were found identical with the previous autopsy and H. strains (table 3).

TABLE 1
Agglutination of H strains (examination I) by Lee serum

STRAINS	SERUM DILUTIONS						CONTROL
	1:50	1:100	1:200	1:400	1:700	1:1000	
H-I-1.....	+	+	+	±	×	—	—
H-I-2.....	+	+	+	±	×	—	—
H-I-3.....	+	+	+	±	×	—	—
H-I-4.....	+	+	+	±	×	—	—
H-I-5.....	+	+	+	±	×	—	—
H-I-6.....	+	+	+	±	×	—	—
H-I-7.....	+	+	+	±	×	—	—
H-I-8.....	+	+	+	±	×	—	—
H-I-9.....	+	+	+	±	×	—	—
H-I-10.....	±	+	+	±	×	—	—
Lee.....	+	+	+	±	×	—	—

+ = Complete agglutination.

± = Marked agglutination.

× = All reactions weaker than those above.

— = No reaction.

TABLE 2
Agglutinin absorption tests with H strains (examination I) and Lee serum

STRAINS	AGGLUTINATION BEFORE ABSORPTION						ABSORBED BY STRAINS	AGGLUTINATION AFTER ABSORPTION												
								Absorbing strain						Serum strain						
	1:50	1:100	1:200	1:400	1:700	1:1000		1:50	1:100	1:200	1:400	1:700	1:1000	1:50	1:100	1:200	1:400	1:700	1:1000	CONTROL
H-I-3	+	+	+	±	×	—	H-I-3	—	—	—	—	—	—	—	—	—	—	—	—	—
H-I-7	+	+	+	±	×	—	H-I-7	—	—	—	—	—	—	—	—	—	—	—	—	—
Lee	+	+	+	±	×	—	Lee	—	—	—	—	—	—	—	—	—	—	—	—	—

Case II. Miss B accidentally sucked into her mouth some salt solution suspension of an eighteen-hour culture on blood agar. She developed symptoms of a pharyngeal infection on the fourth day. There was a slight rise of temperature and moderate prostration. On her return nasopharyngeal cultures yielded influenza bacilli identical

in type with those in the Holmes culture (table 4). Holmes was one of a group of marines among whom influenza developed some weeks before.

Case III. Miss R was accidentally infected with Marine 3 culture, a fourth day case strain isolated from the nasopharynx. She received infection in the same manner as Dr. H. Miss R. was not ill after the

TABLE 3
Agglutination of H strains (examination II) by Lee serum

STRAINS	SERUM DILUTIONS						CONTROL
	1:50	1:100	1:200	1:400	1:700	1:1000	
H-II-1.....	+	+	+	±	×	—	—
H-II-2.....	+	+	±	±	×	—	—
H-II-3.....	+	+	+	±	×	—	—
H-II-4.....	+	+	+	±	×	—	—
H-II-5.....	+	+	+	±	×	—	—
H-II-6.....	+	+	+	±	×	—	—
H-II-7.....	+	+	+	±	×	—	—
H-II-8.....	+	+	+	±	×	—	—
H-II-9.....	+	+	+	±	×	—	—
H-II-10.....	+	+	+	±	×	—	—
H-II-11.....	+	+	+	±	×	—	—
Lee.....	+	+	+	±	×	—	—

TABLE 4
Agglutinin absorption test with B strain and Holmes serum

STRAINS	AGGLUTINATION BEFORE ABSORPTION						ABSORBED BY STRAINS	AGGLUTINATION AFTER ABSORPTION										CONTROL			
								Absorbing strain					Serum strain								
	1:100	1:200	1:400	1:600	1:800	1:1000		1:100	1:200	1:400	1:600	1:800	1:1000	1:100	1:200	1:400	1:600		1:800	1:1000	
B Holmes	+	+	+	+	+	+	B Holmes	—	—	—	—	—	—	—	—	—	—	—	—	—	—

accident. Twelve strains isolated from plate cultures made twenty-eight days after the accident were all alike and were identical with the accident strain (tables 5 and 6).

Case IV. Miss C was accidentally inoculated in a similar manner to Miss R. and Dr. H. but neither developed symptoms nor became a carrier.

TABLE 5
Agglutination of R strains by Marine 3 serum

STRAINS	SERUM DILUTIONS						CONTROL
	1:50	1:100	1:200	1:400	1:700	1:1000	
R-1.....	+	+	+	+	+	±	—
R-2.....	+	+	+	+	+	±	—
R-3.....	+	+	+	+	+	±	—
R-4.....	+	+	+	+	+	±	—
R-5.....	+	+	+	+	+	±	—
R-6.....	+	+	+	+	+	±	—
R-7.....	+	+	+	+	+	±	—
R-8.....	+	+	+	+	±	±	—
R-9.....	+	+	+	+	+	±	—
R-10.....	+	+	+	+	+	±	—
R-11.....	+	+	+	+	+	±	—
R-12.....	+	+	+	+	+	±	—
Marine 3.....	+	+	+	+	+	±	—

TABLE 6
Agglutinin absorption tests with R strains and Marine 3 serum

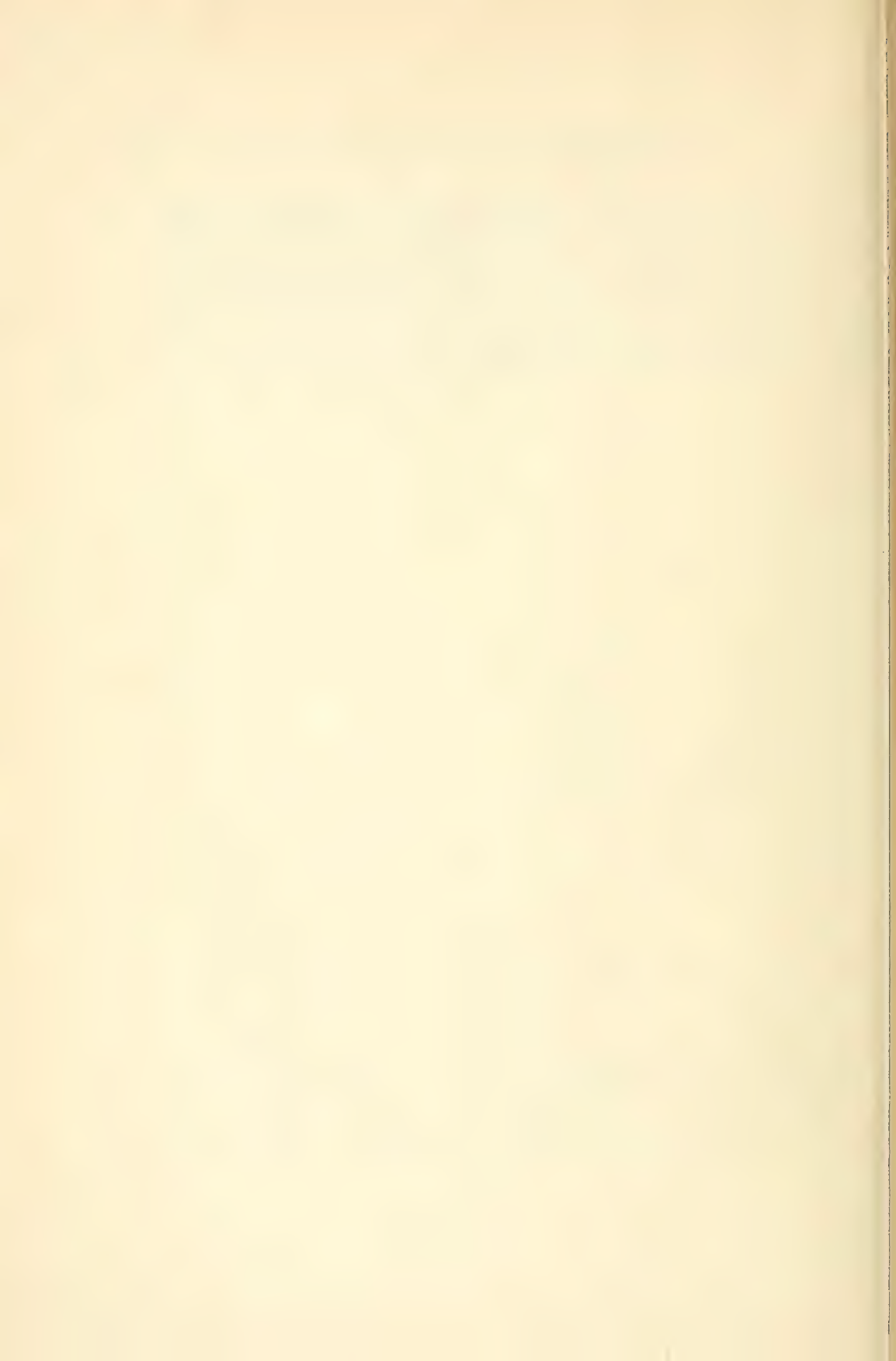
STRAINS	AGGLUTINATION BEFORE ABSORPTION						ABSORBED BY STRAINS	AGGLUTINATION AFTER ABSORPTION										CONTROL	
								Absorbing strains					SERUM STRAIN						
	1:50	1:100	1:200	1:400	1:700	1:1000		1:50	1:100	1:200	1:400	1:700	1:1000	1:50	1:100	1:200	1:400		1:700
R-3	+	+	+	+	+	±	R-3	-	-	-	-	-	-	-	-	-	-	-	-
R-11	+	+	+	+	+	±	R-11	-	-	-	-	-	-	-	-	-	-	-	-
Marine 3	+	+	+	+	+	±	Marine 3	-	-	-	-	-	-	-	-	-	-	-	-

CONCLUSIONS

1. Freshly isolated Pfeiffer bacilli are capable of causing infection when transferred in great numbers to susceptible mucous membranes.

2. Strains recovered from infected persons at varying intervals after the accidental inoculation, revealed agglutinative characteristics identical with those of the strains which had caused the infection. The varying periods cover the time from shortly after infection to convalescence. This is strong evidence that a

strain responsible for an infection would reveal identical characteristics during infection and convalescence in the epidemic cases due to its invasion. If the epidemic of influenza was due to the Pfeiffer bacillus, we would expect the dominant strains to have similar type characteristics. The fact that just the opposite condition exists is strong proof against the primary etiological importance of the Pfeiffer bacillus in epidemic influenza.



VI. A SEROLOGICAL STUDY OF THE BACILLUS OF PFEIFFER

ARTHUR F. COCA AND MARGARET F. KELLEY

From the New York Hospital and the Department of Bacteriology in Cornell University Medical College, New York City¹

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I. THE ETIOLOGICAL RELATION OF THE BACILLUS OF PFEIFFER TO INFLUENZA

The bacteriological studies of the recent pandemic of influenza, also referred to as the "Spanish disease" and "grippe," have resulted in a divided opinion regarding the rôle of Pfeiffer's bacillus in the disease. The investigators have concluded either that this microörganism is the primary cause of the disease or that it is a secondary invader exerting more or less influence on the clinical course of the primary infection.

In Europe, opinion in this regard has been influenced by the degree of success that attended the efforts to cultivate *B. influenzae* from the sputum and organs of the affected individuals. Some failed entirely to demonstrate the organism and some found it in only a few cases. These workers (10, 12, 23) opposed Pfeiffer's view of the significance of his bacillus in influenza and some of them were inclined to the view—which was based on cultural studies and inhalation (spray) experiments in human beings with filtered sputum (1, 4, 26, 35)—that influenza is caused by a filterable virus. Another group of workers (2, 22, 41, 42) found a gram-positive "diplo-streptococcus" or a diplococcus and out of this finding comes a suggested etiological significance of these microörganisms. Other European investigators (8, 9, 16, 21, 24, 25, 26, 36) who isolated *B. influenzae* in a considerable

¹ Aided by grant from the Metropolitan Life Insurance Company Influenza Commission.

proportion of their cases (50 to 75 per cent) offered these findings in support of Pfeiffer's view. A few writers question the use of the negative findings as an argument against the etiological significance of *B. influenzae*, on account of the known difficulty of cultivating that microorganism.

The majority of American investigators have been inclined to favor the view of Pfeiffer, either because of the production of a pathological condition in monkeys resembling influenza by the administration of cultures of *B. influenzae* (5); or because of similar results with other lower animals together with the demonstration of a toxin-like poison in broth cultures of *B. influenzae* (7, 18, 29); or, finally, because of an apparent prophylactic action resulting from the injection into human beings of "vaccine" prepared from cultures of *B. influenzae* (6). Some (30), it is true, are impressed by the frequent finding of *B. influenzae* in influenza.

On the other hand, the idea that the primary cause of influenza is a filterable virus is not without followers in this country.

On the basis of extensive serological studies carried out under his direction, Park (27, 28) has introduced a new argument into the discussion of the question in hand. The argument lies in the self-evident proposition that the most characteristic feature of influenza; namely, its pandemic occurrence, which distinguishes it from all other infectious diseases, requires the assumption of a single etiological factor in its causation. According to this proposition it is not sufficient, in order to prove the causal relationship of Pfeiffer's bacillus to influenza, that an obligative hemophilic, pathogenic bacillus be found in all or nearly all cases of influenza. It is necessary, in addition to this, to show, with the aid of reliable biological reactions, such as that of agglutination, that the cultures obtained from influenza patients that are known not to have been in contact with one another are identical.

It was with this principle in mind that Eugenia Valentine and Georgia M. Cooper (40), under Park's direction, made their study of different cultures of *B. influenzae* obtained from cases of influenza during the recent epidemic. These investigators injected rabbits with cultures of *B. influenzae* and tested each individual antiserum produced against a single culture with the same

(homologous) culture and with other cultures of *B. influenzae*. The tests were made by direct agglutination and by agglutination after absorption with the homologous cultures as well as with the other cultures that showed cross-agglutination.

The results obtained with the use of this method of study were surprising and highly illuminating. Not only were no identities of the cultures found, excepting where previous contact between the individuals concerned could be demonstrated or surmised, but in one family, the six members of which were stricken at very nearly the same time, the six cultures obtained were all different. Thus, even under circumstances most favorable for the finding of a hypothetical pandemic strain or culture of the bacillus of Pfeiffer, the study failed to reveal the existence of any such strain.

In the light of Park's argument these observations seem to admit of but one conclusion; namely, that Pfeiffer's bacillus cannot be the specific cause of influenza.

The importance of this conclusion makes it obviously advisable that the observations on which it is based be confirmed and it was with this purpose in view that the present study was undertaken.

The cultures studied were of six groups as to source.

Cultures Holmes, Eldridge, Gordon and Amert were of the marine group of Valentine and Cooper, who designated the cultures as M7, M3, M4 and M1, respectively. Cultures Leuchner and Meyer were the cultures H11 and H13 of Valentine and Cooper. Cultures Lee, Williams, Masates and Godfrey were autopsy strains designated with these names in the paper of Valentine and Cooper. Cultures Angelo and Michael were derived from the family group of Valentine and Cooper. Cultures 126, 159, 160 and Witt were isolated from cases of influenza during the winter 1919-1920 by Dr. Anna Williams. Cultures 62 and G. L. T. were kindly supplied by Dr. E. O. Jordan of Chicago, these having been isolated from cases of influenza in Chicago during the last winter.

Thus, the cultures subjected to the present examination were derived from widely different sources, both as to the time and as to the locality of their isolation.

CULTURE MEDIUM

Throughout the present study we have used the so-called "chocolate" medium; that is heated blood-agar. The proper method of preparing this medium and the history of the method seem generally to have had inadequate consideration in the literature.

The advantage of heating the mixture of blood and agar was first noted by Dr. Olga Povitzky in an inconspicuous remark made in the course of one of the Collected Studies from the Bureau of Laboratories, Department of Health, City of New York, 1912-13, vii, 93. At the foot of page 94 is found the following statement: "Recently we found that a more vigorous growth is obtained on coagulated blood medium. This medium is obtained by adding blood to agar (1:10 to 1:500) at 90°C."

In a paper entitled "Zur Züchtung des Influenzabazillus," Hundeshagen referred to an "American method" of which he had learned in 1915, but which he could not find in the collected studies referred to, although he had searched through the issues of the "past four years." Hundeshagen employed the method with very good results. He heated the blood-agar mixture at 96°C.

A. Fleming boils the blood-agar mixture for one minute and obtains an "enormous growth" in this slanted mixture.

In our experience the volume of growth of *B. influenzae* on the blood-agar medium is markedly influenced by the temperature to which the mixture has been heated. While no formal experiments upon this point have been carried out, we can say that the temperature which will barely cause the coagulation of the blood, with the production of the chocolate brown color, is not high enough to provide the optimum growth conditions in the medium; these conditions can be secured by subjecting the mixture to a temperature between 96° and 100°C. for a period of ten minutes—possibly less. The mixture can be made in individual tubes, these having been taken out of boiling water and returned to it for the required time; or the mixture can be made

in bulk in a flask immediately after the agar has been removed from the autoclave.

We have employed neutral, nutrient agar containing 5 per cent glycerin, adding 10 per cent of sterile horse's blood.

PRODUCTION OF ANTISERA

Twenty-four-hour cultures were injected intravenously into medium sized rabbits over a period of three weeks, as follows:

On the first day a suspension of one agar slant growth in 2 cc. of sterile saline solution was heated for one hour at 60°C. and of this 1 cc. was injected.

On the 11th, 12th, 13th, 17th, 18th, 19th and 20th days 1 cc. of a similar suspension of the unheated bacteria was injected. On the 25th day the animals were bled and the sera were tested with the method of direct agglutination. If the titer of the serum was found to be lower than 1:640, as it was in two instances, the injections were continued daily for a period of ten days, after which in both cases the titer was found to be satisfactory; that is, 1:1280 and 1:640, respectively.

Antisera were prepared against eight different cultures and each one of these was tested by direct agglutination with all of the eighteen cultures included in this study.

The results of the test by direct agglutination are presented in table 1.

It is seen that most of the sera were able to agglutinate some of the heterologous cultures. However, in most of these instances the cross agglutination was so slight (1:320 or less) that the question of a possible identity of the respective cultures did not need to be considered. Moreover, a later test carried out preliminary to a proposed absorption experiment, showed, in all, a considerable diminution in even this slight degree of cross agglutination, which rendered the absorption experiment entirely superfluous.

The absorption experiment was carried out with the antisera Gordon and Eldridge. The absorptions carried out with the antiserum Holmes are described below in the special study of that serum.

TABLE 1

Direct agglutinations

SERUM DILUTION.....	SERUM ANTI-LEE					SERUM ANTI-MASATES					SERUM ANTI-GODFREY					SERUM ANTI-MICHAEL					SERUM ANTI-GORDON					SERUM f ANTI-HOLMES					SERUM ANTI-ELDRIDGE					SERUM ANTI-WILLIAMS				
	80	160	320	640	1280	2560	80	160	320	640	1280	2560	80	160	320	640	1280	2560	80	160	320	640	1280	2560	80	160	320	640	1280	2560	80	160	320	640	1280	2560				
Lee.....	+	+	+	+	+	0	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Masates.....	0	0	0	0	0	0	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Godfrey.....	0	0	0	0	0	0	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Michael.....	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Gordon.....	0	0	0	0	0	0	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Holmes.....	0	0	0	0	0	0	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Eldridge.....	0	0	0	0	0	0	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Williams.....	0	0	0	0	0	0	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Leuchner.....	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Amert.....	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Angela.....	×	×	×	×	×	×	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Meyer.....	0	0	0	0	0	0	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
126.....	0	0	0	0	0	0	×	×	×	×	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
159.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
160.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Witt.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
G. L. T.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
62.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			

+ = complete agglutination; ± = partial agglutination; × = slight agglutination; 0 = no agglutination.

TECHNIC

The bacterial sediment used in the absorption experiment represented a twenty-four-hour "chocolate-agar" slant growth which had been planted from a previous twenty-four-hour chocolate-agar culture. The bacteria were scraped off the surface of the medium, suspended in about 10 cc. of sterile saline solution and thrown down in the centrifuge.

After decantation of the supernatant fluid there was added to the sediment a quantity of the serum to be absorbed equal to one-half of the volume of the bacterial sediment. The serum

TABLE 2
Agglutinin absorptions

SERUM	AGGLUTINATION BEFORE ABSORPTION							ABSORBED BY STRAIN	AGGLUTINATION AFTER ABSORPTION												
	Strains								Of absorbing strain					Of homologous strain							
		80	160	320	640	1280	2560		Control	80	160	320	640	1280	2560	80	160	320	640	1280	2560
Gordon . . .	Gordon Amert	+	+	+	+	+	×	0	Gordon Amert	0	0	0	0	0	0	0	0	0	0	0	0
Eldridge . . .	Eldridge	+	+	+	+	0	0	0	Eldridge	0	0	0	0	0	0	0	0	0	0	0	0
	Holmes	+	+	+	+	±	0	0	Holmes	0	0	0	0	0	0	0	0	0	0	0	0
	Godfrey	+	+	+	+	±	0	0	Godfrey	0	0	0	0	0	0	+	+	+	±	0	0

and bacterial sediment were mixed with a quantity of sterile saline solution necessary to provide a 1:16 dilution of the serum. The mixture was placed in a water-bath at 45°C. for several hours and afterwards in the ice-box over night. The supernatant fluid was tested with suspensions of twenty-four-hour growths of the cultures under consideration.

The results of the absorptions that were carried out are shown in table 2.

It is seen that the cultures Gordon and Amert are identical, this finding confirming that of Valentine and Cooper. Cultures Eldridge and Holmes seem, by the test, to be identical, while Godfrey is different. We have carried out similar absorptions

with two sera prepared against cultures Eldridge and Holmes by Miss Valentine and Miss Cooper and with both of these sera, also, we obtained nearly complete cross absorption.

In view of the discordance between our findings and those of Valentine and Cooper with respect to these two cultures, we have immunized another rabbit with culture Holmes and we have carried out absorptions with the resulting agglutinating serum. This serum agglutinated cultures Holmes and Godfrey in a dilution of 1:1280 and it agglutinated culture Eldridge in a dilution of 1:640. After absorption with culture Godfrey the serum agglutinated both cultures Holmes and Eldridge but not Godfrey. After absorption with culture Eldridge the serum agglutinated culture Holmes in a dilution of 1:1280, but could not agglutinate either culture Eldridge or culture Godfrey.

We are unable to explain these discordant results in the different absorption experiments. However, the discordance is immaterial to the main argument since the individuals from whom the cultures were obtained had been in personal contact with each other.

The foregoing study confirms the findings of Valentine and Cooper and supports the conclusion of Park that the bacillus of Pfeiffer can not be the cause of influenza.

The non-identity of different cultures of Pfeiffer's bacillus has been recognized elsewhere. Beiling found identity in two strains out of six, all of undescribed source. A paper has just been published by H. H. Bell (1a) in which this author, in a study of a large number of cultures obtained from cases of influenza in the St. Louis Children's Hospital, completely confirms the findings of Valentine and Cooper.

Neither of these investigators seems to have realized the significance of these findings with regard to the question of the etiology of influenza. Bieling indicates his belief in the etiological relationship of the bacillus of Pfeiffer to influenza by adopting the suggestion of Novacovic and of Neufeld and Papamarku that a polyvalent antigen of *B. influenzae* be used for the diagnosis of influenza (Widal technic).

II. AN UNUSUAL OBSERVATION UPON THE NATURAL INHIBITION IN AGGLUTINATING SERUM

In table 1 it is seen that the serum obtained after immunization with culture Holmes was able to agglutinate both cultures Angela and Godfrey in a dilution of 1:1280, but it could not clump its homologous culture in any concentration within the usual limits. This phenomenon has not been hitherto observed, or at least recognized, perhaps on account of the fact that the preliminary examination of an antibacterial serum is usually carried out with only the homologous culture.

In order to exclude the possibility of a technical error, we tested the serum again by direct agglutination with the cultures Holmes, Godfrey and Angela and at the same time we assured ourselves of the identity and agglutinability of our Holmes culture by testing it with two anti-Holmes sera prepared by Miss Valentine and Miss Cooper. The results of this retest were identical with those obtained at the first examination.

Two possible explanations of the phenomenon presented themselves: Either no major agglutinins had been produced in the injected rabbit or the agglutinins which had been formed against the injected culture were prevented from exhibiting their usual effect on account of the action of an inhibiting mechanism. The former possibility could not be immediately investigated; the latter was subjected at once to experiment.

The tests already described gave evidence that if the lacking agglutination of the homologous culture was due to an inhibiting mechanism, the inhibition was specific, since it did not interfere with the group agglutinations. The actual demonstration of the inhibiting mechanism was made in an experiment the results of which are presented in table 3.

In two series of test tubes were distributed constant quantities (two completely agglutinating doses) of two different anti-Holmes sera (Valentine and Cooper). To each tube of the two series were added the usual quantity of a suspension of a twenty-four-hour blood-agar Holmes culture and diminishing quantities of our anti-Holmes serum f. In both series agglutination was completely inhibited by $\frac{1}{320}$ cc. of the serum f.

A further test of the specificity of this inhibition was made by mixing the serum f with two completely agglutinating doses of the anti-sera Gordon, Masates and Michaels; these doses were respectively $\frac{1}{1280}$, $\frac{1}{320}$ and $\frac{1}{320}$ cc. In no case was agglutination in the least inhibited by as much as 0.1 cc. of undiluted serum f, or by any smaller quantity down to $\frac{1}{320}$ cc.

The existence of an inhibiting mechanism was thus demonstrated and it seemed probable, therefore, that there were major

TABLE 3
Showing the inhibiting property of serum f

	TUBE 1	TUBE 2	TUBE 3	TUBE 4	TUBE 5	TUBE 6	TUBE 7	TUBE 8
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
<i>Series 1</i>								
Serum 1108 diluted 1:16.....	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Serum f undiluted....	$\frac{1}{16}$	$\frac{1}{20}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$	$\frac{1}{1280}$	$\frac{1}{2560}$	$\frac{1}{5120}$
Suspension of Holmes culture.....	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Saline solution.....	Up to a total volume of 1 cc.							
Results after twenty-four hours at 45°C..	0	0	0	0	?	+	+	+
<i>Series 2</i>								
Serum 725 diluted 1:16	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Serum f undiluted....	$\frac{1}{16}$	$\frac{1}{20}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$	$\frac{1}{1280}$	$\frac{1}{2560}$	$\frac{1}{5120}$
Suspension of Holmes culture.....	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Saline solution.....	Up to a total volume of 1 cc.							
Results after two hours at 45°C.....	0	0	0	0	+	+	+	+

agglutinins in the serum the action of which was prevented. That this is true, was proven by the fact that upon several weeks standing the serum f rather abruptly acquired the ability to agglutinate the homologous culture. In table 4 are shown the results of the agglutination tests that were carried out on the 37th, 40th and 50th days after the serum had been obtained from the rabbit.

Two possibilities presented themselves in explanation of the inhibition. Either the agglutinins were prevented from uniting

with the homologous bacteria or after such union had taken place the usual clumping was inhibited. This question was investigated with the technic of absorption.

With 0.25 cc. of the bacterial sediment of a twenty-four hour blood agar growth of the Holmes culture were mixed 0.1 cc. of the serum f and 1.4 cc. of sterile 1 per cent saline solution. After this mixture had stood at 45°C. for four hours and in the ice-box overnight the supernatant fluid obtained by centrifugation was tested with the homologous strain and with all of the heterologous strains which the unabsorbed serum was capable of clumping. All of these tests resulted negatively, all agglutinating power having been removed.

TABLE 4

Showing the disappearance of the inhibiting property of serum f

DAY OF TEST	DEGREE OF AGGLUTINATION PRODUCED UPON CULTURE HOLMES BY SERUM F					
	Quantity of serum f					
	$\frac{1}{160}$ cc.	$\frac{3}{320}$ cc.	$\frac{6}{640}$ cc.	$\frac{12}{1280}$ cc.	$\frac{25}{2560}$ cc.	$\frac{51}{5120}$ cc.
37th	=	=	=	=	=	=
40th	+	++	+++	+++	+	=
50th*	++++	++++	++++	++++	++	=

* On this day the serum was found, also, to cause complete agglutination in all quantities between $\frac{2}{160}$ cc. and $\frac{1}{810}$ cc.

As the homologous agglutination by the supernatant fluid could conceivably have been prevented by the continued presence of the inhibiting mechanism a second absorption experiment was carried out and this time the supernatant fluid was examined for the presence of that mechanism. The results of this experiment are presented in table 5.

It is seen that at least 93.75 per cent of the inhibiting power of the serum had been removed by the bacterial sediment. The experiment demonstrates, therefore, that the inhibition is due to the presence of a specific substance which is absorbed by the homologous culture and prevents the clumping action of the homologous agglutinins, which also are absorbed. The experiment was carried out on the 37th day of the serum's life, at which

time, as we have seen above, the inhibiting power of the serum was already diminishing.

The foregoing study has produced some observations which may throw light on the mechanism of the inhibition often observed in the use of the larger quantities of agglutinating immune sera. In their well-known work upon this subject Eisenberg and Volk came to the conclusion that that inhibition is due to

TABLE 5

Showing the absorption of the inhibiting substance in serum f by the homologous bacteria

A mixture of 0.1 cc. of serum f, 0.2 cc. of bacterial sediment of the Holmes culture and enough of a 1 per cent saline solution to make a total volume of 1.7 cc. was allowed to stand for two hours at 45°C. and overnight in the ice-box. The supernatant fluid, obtained by centrifugation, was compared with the unabsorbed serum f as to its inhibiting power.

	TUBE 1	TUBE 2	TUBE 3	TUBE 4	TUBE 5	TUBE 6
	cc.	cc.	cc.	cc.	cc.	cc.
<i>Series 1</i>						
Supernatant fluid.....	0.2	0.1	0.05	0.025	0.0125	
Suspension of Holmes culture.....	0.5	0.5	0.5	0.5	0.5	
Serum 1108 or 725.....	$\frac{1}{320}$	$\frac{1}{320}$	$\frac{1}{320}$	$\frac{1}{320}$	$\frac{1}{320}$	
Result { 1108	++++	++++	++++	++++	++++	
(agglutination) .. { 725	++++	++++	++++	++++	++++	
<i>Series 2 (control)</i>						
Serum f.....	$\frac{1}{40}$	$\frac{1}{50}$	$\frac{1}{60}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{240}$
Suspension of Holmes culture.....	0.5	0.5	0.5	0.5	0.5	0.5
Serum 1108 or 725.....	$\frac{1}{320}$	$\frac{1}{320}$	$\frac{1}{320}$	$\frac{1}{320}$	$\frac{1}{320}$	$\frac{1}{320}$
Result { 1108	=	=	+	++	++	+++
(agglutination) .. { 725	0	=	=	=	++	++++

the action of altered agglutinins—agglutinoids. Such altered agglutinins were supposed by these authors to lack the power of agglutination while possessing a uniting power (binding group) in enhanced degree (increased avidity). A similarly acting property of inhibition could be induced in agglutinating sera by heating or by ageing. The inhibition was not directly demonstrable in the aged serum, but it was inferred by the authors in explanation of an altered coefficient of absorption.

It seems likely that the inhibiting substance of our serum f was unique, not in its quality but in its quantity in the serum. Serum f, in other words, contained an unusually large quantity of the natural inhibiting substance of agglutinating sera.

A striking difference is manifest in our observations between this natural inhibiting substance and the artificial "agglutinoid" described by Eisenberg and Volk. This difference lies in the fact that while "agglutinoid" is formed in agglutinating serum upon standing, the inhibiting substance of serum f lost its power of inhibition completely on standing.

We are unable to say whether this instability is a property of the natural inhibiting substance of all agglutinating sera. However, we can record that in one serum (Eldridge) the inhibiting power that was exhibited only by the larger quantities (0.2 cc. to 0.05 cc.) was completely lost by the 21st day.

In view of the radical difference just referred to, there seems to be no ground for looking upon the natural inhibiting substance as a modified agglutinin. That it is a specific antibody is clear. Its relation to the inhibiting mechanism of the Neisser-Wechsberg phenomenon has not been investigated.

The assumption that the inhibiting substance has a greater "avidity" than the active agglutinins seems superfluous, in view of the fact, demonstrated by Eisenberg and Volk themselves, that the previous absorption of the inhibiting substance by the bacteria does not prevent the subsequent attachment of agglutinins to the same bacteria. Our own experiments show that the inhibiting and the agglutinating antibodies are absorbed together.

SUMMARY

1. Confirming the previous work of Valentine and Cooper, a study of eighteen cultures of the bacillus of Pfeiffer isolated from cases of influenza in different localities and at different times revealed identities in the cultures only when a probability of personal contact existed.

2. On the basis of Park's argument these findings admit of only one conclusion with regard to the rôle of Pfeiffer's bacillus; namely, that that microorganism is not the cause of the disease.

3. An immune serum prepared by the injection of one strain of *B. influenzae* was found to agglutinate other strains of that bacillus, but not the one used for the immunization. This phenomenon was due to the presence of an unusual quantity of a specific inhibiting antibody.

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VII. REPORT ON THE PROPHYLACTIC VACCINATION OF 1536 PERSONS AGAINST ACUTE RESPIRA- TORY DISEASES, 1919-1920

ANNA I. VON SHOLLY AND WILLIAM H. PARK

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The reason for the investigation upon which this report is based, was to attempt to help clear up, if possible, the status of a vaccine made of the mixed organisms which predominate in respiratory diseases, used as a prophylactic agent in epidemic "grippe" and acute respiratory diseases in general. Reports on vaccine prophylaxis are only too plentiful both for a vaccine made of pure *Bacillus influenzae* and for a vaccine made of various mixtures of the important microbes found in the respiratory tract. The chief criticisms to the recognition of their claims is that they were tested out during the late pandemic of "grippe" either close to or after the peak of the epidemic, at a time when it was impossible to sort out cases into convincing statistical groups on account of the unknown factor of natural or inherited immunity which plays an important and as yet, little charted part in epidemiology. Because, too, of this unknown denominator of natural immunity, adequate control groups, for scientific comparison with the inoculated groups, during the epidemic period, were not obtainable.

Our aim, then, in this study, was to inoculate prophylactically a group of people well in advance of any anticipated recurrence of epidemic grippe so that whatever immunity might be stimulated by this method would be already established before exposure to the disease. Moreover, we wished to learn what effect a vaccine made of the predominant organisms of the acute respiratory diseases would have in inhibiting or modifying these diseases. We aimed, too, to have as a comparison an uninoculated control group as similar as possible to the group inoculated.

The work presented was done between September 30, 1919, and April 3, 1920, among the general employees of the Metropolitan Life Insurance Company's Home Office, New York City, under the direction of the Research Laboratory of the New York City Health Department, coöperating with the officers of the Metropolitan Life Insurance Company. This company gave the services of its nursing and clerical forces and defrayed the cost of the vaccine. The groups under observation consisted of men and women, all volunteers, of varying ages. In all, there were 1536 people who were inoculated, of whom 1293 received three inoculations, 95, two inoculations, and 148, only one inoculation. As a control group 3025 persons of similar ages and both sexes of the same company, were under continuous observation. Only about 13 per cent of the original inoculated group and 14 per cent of the uninoculated dropped out during the six months of investigation.

The personal history, home conditions and habits of the entire group as given in the original form filled out by each volunteer at the beginning of our work, summarized in percentages taken from 1500 histories taken at random shows similarity between the two subgroups; i.e., inoculated and uninoculated.

METHOD OF FOLLOWING THE GROUPS

On entering the group, each person filled out a form, which was kept under his or her name in a filing cabinet. All subsequent records of each person were attached to this form as they were collected. That we might not slip up in following the course of each one's health, we imposed a series of checks in the collection of the material. A form was given each person to fill out and return when he or she was suffering from any acute respiratory disease. Still another form was given section heads who were made responsible for their weekly return to us with the names and condition of those in the section suffering from "colds." In addition, copies were made daily from the dispensary records of all persons who had reported for treatment for any respiratory disease during that same day. For several months, many of

these people were seen personally by one of us (von Sholly) in order to obtain material for culture work. Furthermore, from the Medical Welfare Bureau, which has supervision over all employees who are absent for illness, we obtained daily reports, with diagnoses of the disease and length of absence. Pneumonia cases were investigated to confirm them. All the facts obtained were inscribed daily on the records and filed away. To indicate the extent of the statistical work, we may state that we had one doctor, one nurse, two whole time clerks, two part time clerks for a fairly long period, and one laboratory assistant at work daily, for more than six months. We do not consider our records perfect. They could not be so, where one had to deal with a freely moving volunteer group of varying degrees of intelligence and coöperation. We hope, however, that they are better than many that have been offered. We undoubtedly did not get a record of every "cold" experienced in the group but feel confident that whatever errors have crept in were true of both groups, inoculated and not inoculated, and that, with the check of the absentee and the dispensary reports, only the mildest affections requiring slight treatment and no absence are not recorded. The individual diagnoses, too, may not be strictly accurate. Some were self diagnosed (checked as far as possible by the Company's physician). Furthermore, the diagnoses of outside physicians may not conform absolutely to our nomenclature. By grouping the infections, however, into upper and lower respiratory diseases, we think they are fairly accurate—sufficiently so for the purposes of this investigation.

The injections were given at weekly intervals during the end of September, 1919, and during the first three weeks of October, 1919. Two vaccines¹ were used. The larger group of persons numbering 1412, were given vaccine *L*, a saline suspension sterilized by heat and made up of:

¹ These formulæ were selected by a committee composed of Dr. Park, Dr. Rosenau of Harvard, Dr. McCoy of the United States Public Health Service, and Professor Jordan of Chicago University.

	million per cc.
<i>B. influenzae</i>	1000
<i>Streptococcus hem</i>	1000
<i>Streptococcus vir</i>	1000
Pneumococcus 1.....	2000
Pneumococcus 2.....	2000
Pneumococcus 3.....	1000

The smaller group, numbering 124, were given vaccine *R*, a vaccine similar in composition to that given by Rosenow of the Mayo Clinic. It consisted of:

	millions per cc.
Pneumococci type 1 (10 per cent), 2 (14 per cent), 3 (6 per cent)...	1500
Pneumococci, group 4 and allied green producing	
Diplostreptococci.....	1500
Hemolytic streptococci.....	1000
Staphylococci (aureus).....	500
<i>B. influenzae</i>	500

We kept a record of the reactions to the vaccines given, partly for general scientific knowledge and partly for the purpose of finding out the practicability of group vaccination for a commercial company from the point of view of loss of services following and due to the inoculations. The injections were given subcutaneously in the deltoid region. The first dose was 0.5 cc. and the second and third were 1 cc. each.

There were, following the first injection, mild local reactions (area of redness not more than a silver dollar in size, and very slight soreness) among 63.8 per cent of the group; moderate local reactions, (area of redness from size of a silver dollar to size of hand and slight soreness) in 11.8 per cent of group; marked local reactions, (anything larger than moderate) in 3.3 per cent of the group and no reaction at all in 21 per cent. Following the second injection (double the dose) mild local reactions were observed in 63.2 per cent of the group, moderate in 21.4 per cent, marked in 2.7 per cent and no reactions in 12.7 per cent. Following the third injection, mild local reactions were seen in 60.6 per cent, moderate in 9.7 per cent, marked in 4.1 per cent, and none in 25.5 per cent.

Of the general reactions, the mild ones, consisting of headaches, slight dizziness, nausea or general malaise were reported after

the first injection, in 19.4 per cent of the group; after the second injection in 27 per cent and after the third in 23.7 per cent. The marked reactions, chills or chilly feelings, fever, general pains, or mild prostration, after the first injection, were observed in 3.1 per cent; after the second, in 3.2 per cent and after the third, among 2.6 per cent. Only 26.2 per cent of the total inoculated gave general reactions. These figures show that the largest number of reactions, as was to be anticipated, followed the second injection and that the severe reactions were comparatively few. There were no really alarming reactions. The details of the reactions of the vaccines are given in table 1.

TABLE 1

Number and percentage of persons showing local and general reactions of specified degree of severity for each inoculation of vaccine

VACCINE, REACTION	ALL INOCULATIONS		INOCULATION ONE		INOCULATION TWO		INOCULATION THREE	
	Num-ber	Per-cent	Num-ber	Per-cent	Num-ber	Per-cent	Num-ber	Per-cent
Both vaccines								
Total.....	4217	100.0	1536	100.0	1388	100.0	1293	100.0
Local reaction:								
Mild.....	2641	62.6	980	63.8	877	63.2	784	60.6
Moderate.....	605	14.3	182	11.8	297	21.4	126	9.7
Marked.....	141	3.3	51	3.3	37	2.7	53	4.1
None.....	830	19.7	320	20.0	177	12.7	330	25.5
General reaction								
Mild.....	979	23.2	298	19.4	375	27.0	306	23.7
Marked.....	127	3.0	48	3.1	45	3.2	34	2.6

Several persons reported diarrhoea and a few, vomiting which they ascribed to the inoculations. One had a marked general urticaria after the first injection and further inoculations were not given. A certain number had a "feeling as if they had taken cold" or else had a very mild coryza of short duration for a few hours or a day. Another very small group reported a "cold" following the first or second injection which they claimed lasted several weeks or even almost all winter. Whether these "colds" bore any relation to the inoculations is questionable. Practi-

cally all of these cases who were examined were suffering from a chronic catarrhal condition of the naso-pharynx.

That some people should take advantage of the inoculations to remain at home would not be at all surprising, especially, where there is an illness insurance. If we give everybody the benefit of the doubt and count all absences immediately following vaccination as due to it, there were in all 87 persons who were absent from work anywhere from a half day to 24 days. The total days absence of this group was $181\frac{1}{2}$ days, or 2 days and 42 minutes for each one. The average absence per person for the total inoculated group, 1536 persons, taking the working day as 8 hours, was about 56 minutes. We may compare these percentages with the absences due to respiratory diseases during the six months observation period. The uninoculated lost 8172 days or an average of 2.2 days per person and the inoculated lost 2456 days or an average of 1.59 days per person during the same interval. If we add the average of the inoculated persons' absence due to respiratory disease to the average absence credited to inoculations, we still find a gain of a good half working day per person in favor of the inoculated. It is fair also to assume that if the custom of annual vaccinations were established, absences due to inoculations would diminish.

A tabulation was made to learn whether there was any demonstrable relationship between past respiratory diseases, such as pneumonia, bronchitis, grippe, etc., and the character and severity of the reactions from the inoculations. No especial susceptibility to vaccines can be established for these diseases. The same was true for the "allergic" diseases, asthma, hay fever, etc., but the numbers in our group are probably too small to show anything (table 2).

RESPIRATORY AFFECTIONS AMONG THE VACCINATED AND UNVACCINATED DURING THE SIX MONTHS OBSERVATION PERIOD
OCTOBER 1, 1919, TO APRIL 3, 1920

There were 1327 completed records of inoculated persons under continuous observation during the above period and 3025 similar records of the uninoculated. Among the inoculated, 13.7 per

cent give a history of *no respiratory affections* during this time. Among the non-inoculated, 29.77 give a similar history.

TABLE 3

Comparison of respiratory history of active Home Office list cases during winter of 1919-1920 with previous winters. Inoculated and uninoculated persons distinguished. Tabulated by diseases. Both vaccines combined*

RESPIRATORY HISTORY	ALL INOCULATIONS				NO INOCULATION			
	Winter 1919-1920		Past winters		Winter 1919-1920		Past winters	
	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
Total persons.....	1327		1323		3025		2920	
Total cases of respiratory disease.....	1839	100.00	1529	100.00	3826	100.00	3153	100.00
Total number of cases per person.....	1.39		1.16		1.26		1.08	
Upper respiratory infections; total	1185	64.44	1050	68.67	1902	49.71	1959	62.13
Once during period	774	42.08	551	36.04	1481	38.71	1402	44.47
Twice during period.....	255	13.87	278	18.18	294	7.68	371	11.77
Three or more times during period.....	156	8.48	221	14.45	127	3.21	186	5.90
Total upper Colds, coryza and rhinitis..	766	41.65	945	61.81	1156	30.21	1721	54.58
Once.....	441	23.98	484	32.31	851	22.24	1241	39.36
Twice.....	191	10.39	250	16.35	200	5.23	322	10.21
Three or more times.....	134	7.29	201	12.15	105	2.74	158	5.01
Other upper respiratory infections.....	419	22.78	105	6.87	746	19.50	238	7.55
Once.....	333	18.11	57	3.73	650	16.89	161	5.11
Twice.....	64	3.48	28	1.83	74	1.93	49	1.55
Three or more times.....	22	1.20	20	1.31	22	0.58	28	0.89

* All figures do not add up to total of persons, as a number of persons suffered from both upper and lower respiratory diseases during the course of the investigation—1919-1920.

TABLE 3—Continued

RESPIRATORY HISTORY	ALL INOCULATIONS				NO INOCULATION			
	Winter 1919-1920		Past winters		Winter 1919-1920		Past winters	
	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
Lower respiratory infections: total..	187	10.17	91	5.95	308	8.08	174	5.52
Once.....	165	8.97	75	4.91	290	7.58	158	5.01
Twice.....	19	1.03	11	0.72	12	0.31	12	0.38
Three or more times.....	3	0.16	5	0.33	6	0.17	4	0.13
Total lower								
Bronchitis; tracheitis; bronchial cold....	176	9.57	35	2.29	286	7.48	48	1.52
Once.....	154	8.37	23	1.50	268	7.00	34	1.08
Twice.....	19	1.03	7	0.46	12	0.31	10	0.32
Three or more times.....	3	0.16	5	0.33	6	0.17	4	0.13
Pneumonia.....	1	0.05	54	3.53	11	0.29	125	3.96
Once.....	1	0.05	50	3.27	11	0.29	123	3.90
Twice.....			4	0.26			2	0.06
Three or more times								
Other lower respiratory infections (pleurisy, empyema, etc.).	10	0.54	2	0.13	11	0.29	1	0.03
No respiratory infections.....	251	13.65	129	8.44	1139	29.77	553	17.54
Influenza: "grippe"	216	11.75	259	16.94	477	12.47	467	14.81
Once.....	205	11.15	244	15.96	465	12.15	452	14.33
Twice.....	11	0.60	15	0.98	12	0.31	15	0.48
Three or more times								

The number of respiratory infections which occurred in each group and their severity are tabulated in table 3.

One might infer from the tabulated figures that it were wiser not to be inoculated. One must remember, however, that the observed groups were volunteers. Naturally, those volunteering for inoculation might be supposed to be the more susceptible to respiratory diseases, hoping for relief from the inoculations. And, in fact, our summaries of the original questionnaire show that 21.6 per cent of the non-inoculated were comparatively free from respiratory troubles in their past, as against 10.5 per cent of those who submitted to inoculation.

It is probably fair to conclude that, considering the results as a whole, the kind of vaccine used by us, in the manner and dosage indicated, had very little specific influence either in preventing or causing respiratory diseases other than pneumonia.

PNEUMONIA

The difference of pneumonia incidence, however, is marked in the two groups.

The inoculated show 1 case (0.075 per cent) against 11 cases (0.36 per cent) among the non-inoculated, or about five times as many pneumonias among the non-inoculated.

It is true that the number of cases of pneumonia occurring in the group is so small that the apparently favorable results of vaccination may be due to chance, nevertheless, it would seem fair to make some claims for the efficacy of a typed pneumo-streptococcus vaccine as a preventive of pneumonia. The one case of pneumonia which occurred in the inoculated group, was a girl who had had only one injection of the vaccine on October 2, 1919. She suffered from influenza and broncho-pneumonia March 23, 1920, more than five months after vaccination. She was absent from her work just one month. She had had an acute bronchitis (absent 20 days) in January, 1920. Lister (1) in South Africa does not claim much immunity resulting from the pneumococcus vaccine for more than four to six months. After our investigation had been closed, two more cases of pneumonia were reported among the inoculated. Both occurred in May, 1920, about seven months after the vaccinations. One of these patients had had two and the other three injections.

Of the 11 cases of pneumonia occurring among the non-inoculated, before April 3, 1920, 7 were lobar in type and 4 were broncho-pneumonia. Two of the lobar pneumonias and two of the broncho-pneumonias were said to complicate grippe. One case of lobar pneumonia (primary) died after three days illness. In May, 1920, a twelfth pneumonia was reported also among the non-inoculated. If we count these later cases, the percentage was still twice as high for pneumonia among the non-inoculated persons.

GRIPPE CASES

One of the most important phases of our investigation was the completion of the vaccinations about three months before the brief flare up of grippe in January and February, 1920. In the inoculated group, there were 232 grippe cases² during the six months, most of them occurring during January and February. These made up *17.8 per cent* of the inoculated group. Of these cases 49 (*21.1 per cent*) had had grippe in the pandemic of the previous year (1918-1919), 191 of the 232 had been inoculated three times (in October, 1919), 14 had received only two injections and 26 only one injection.

Among the uninoculated, there were 494 cases of grippe, excluding the pneumonias, reported in the period of observation. These were *16.3 per cent* of the group. Eighty-eight persons (*17.8 per cent*) had had influenza also during the previous year. In comparing these groups, we find very little difference in their respective degrees of immunity to what was considered as a recurrent outbreak of influenza.

Looking at these same figures from the point of view of the 1918-1919 epidemic forward to the 1920 return, the results are practically the same. Of the group reporting grippe in 1918-1919, *12.2 per cent* had a second attack in 1920 after inoculation, and *13.1 per cent* had a second attack, although not inoculated.

Most of the grippe cases of 1920 were comparatively mild. The inoculated averaged 7.2 days absence per person, and the non-

² After these figures were obtained, there were a few doubtful cases subtracted—which however made practically no difference in the relative percentages.

inoculated averaged 9.2 days per person. This difference can be accounted for only to a slight degree by the fact that the pneumonias occurred more frequently among the non-inoculated.

SEVERITY OF ILLNESS

It was rather a difficult matter to gauge accurately the severity of the various infections. One had to rely partly on the personal opinion of the patient. However, we made an attempt to judge by an arbitrary classification, as follows: Illnesses were considered severe that showed (1) fever over 102° , (2) confinement in bed four days or more, (3) marked specific clinical symptoms, (4) some prostration. Moderate illnesses were those with (1) fever 100° to 102° , (2) confinement in bed or at home two to four days, (3) moderately severe specific clinical symptoms, (4) malaise. Mild infections were those with (1) a temperature less than 100° , (2) absence less than two days, (3) moderate or mild specific clinical symptoms, (4) slight or no malaise. Our statistics for severity of disease were taken from the absentee reports which, obviously, were the less difficult to judge, since all absentees are visited in their homes by the Company's nurse if they are away more than three days, and all are seen by a nurse or the Company's doctor on their return to work.

In the inoculated group, 445 (30.8 per cent) were absent from October 30, 1919 to April 3, 1920. In the not inoculated group, 1235 (36.1 per cent) were absent. If we divide these absentees into groups according to the degree of severity of their illness, we find 70.8 per cent of both the inoculated and the uninoculated fall into the mild class; 26.7 per cent of the inoculated against 25.7 per cent of the non-inoculated fall into the moderate class, and 2.4 per cent of the inoculated against 3.4 per cent of the uninoculated fall into the severe class.

From the examination of the foregoing statistics, we may draw contrary conclusions depending upon whichever part of the report we lay more stress. If we compare the incidence of the respiratory affections among the inoculated and the uninoculated, excluding pneumonia, and if we consider the severity of these respiratory

affections, we may assert that the vaccines were of little or no appreciable value. If, on the other hand, we focus our attention on the figures that show a somewhat higher susceptibility to respiratory diseases among the vaccinated in their past histories; a larger group absence for respiratory disease among the not vaccinated and a longer average absence per person for attacks of "grippe" in the not inoculated group, we may conclude that the vaccines had a beneficial influence. On the whole, balancing both sides, our evidence does not make a strong case in favor of the vaccines given by us as a prophylactic agent against acute

TABLE 4

Severity of respiratory diseases among absentees on account of sickness

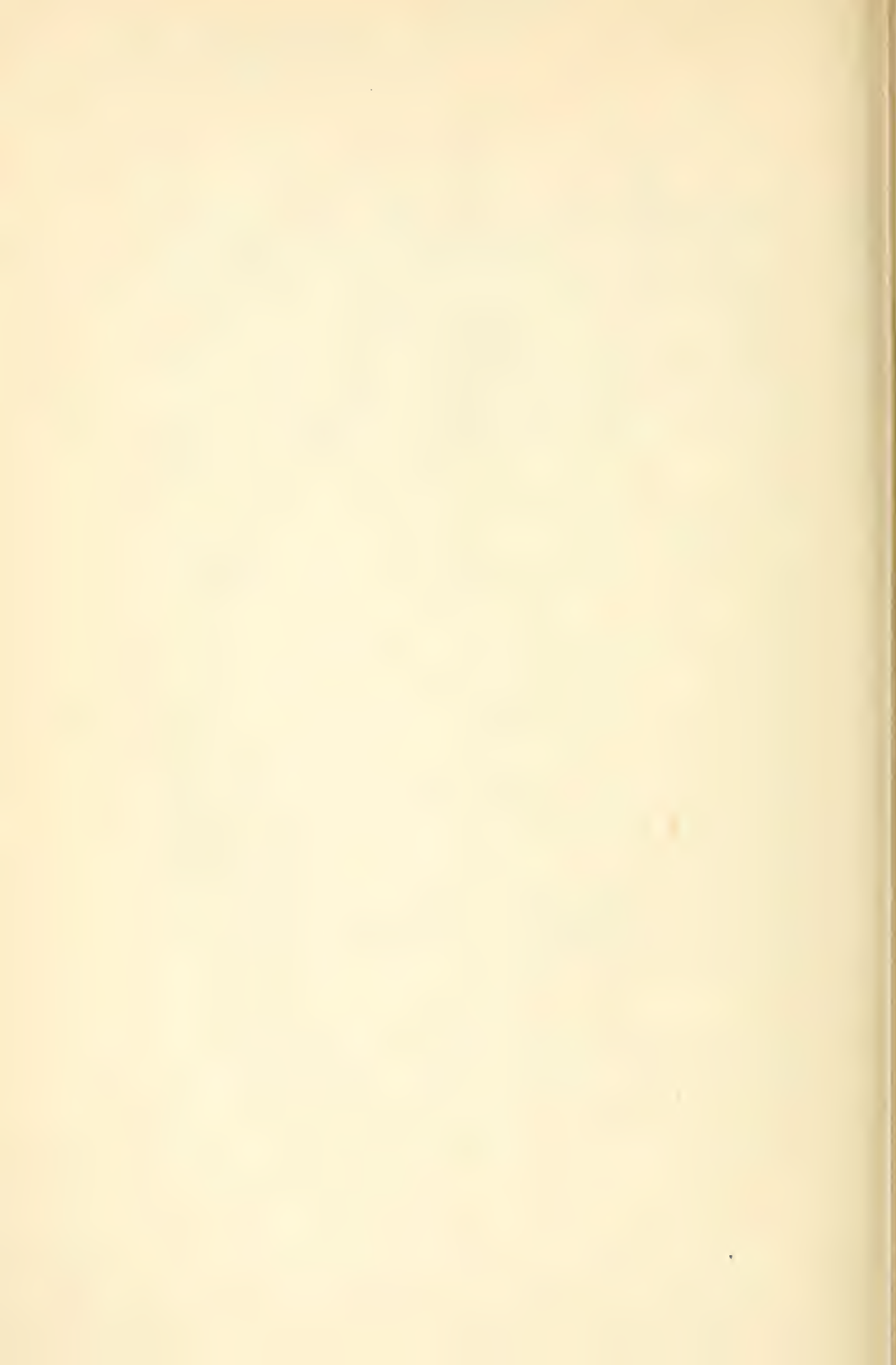
MILD AFFECTIONS	MODERATE AFFECTIONS	SEVERE AFFECTIONS	TOTAL PERSONS	PER CENT WHOLE GROUPS ABSENT FOR RESPIRATORY SICKNESS
Inoculated group				
315 (70.8%)	119 (26.7%)	11 (2.47%)	445 (100%)	30.8%
Not inoculated group				
874 (70.8%)	317 (25.7%)	42 (3.4%)	1235 (100%)	36.1%

respiratory diseases—pneumonia alone excepted. Our work tends to confirm the conclusions of Lister (1) and Cecil and Austin (2) on the value of a typed pneumococcus vaccine for pneumonia. It points to the importance of further research along this line.

Thanks are due for the help and courtesy extended to us by the staffs of the medical and statistical departments of the Metropolitan Life Insurance Company.

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ANTIBODY STUDIES¹

I. REVERSAL OF THE ANTIGEN-ANTIBODY REACTION

F. M. HUNTOON

From the Mulford Biological Laboratories

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INTRODUCTION

Since it was first recognized that certain substances, such as bacteria or their products or certain other poisonous substances of animal and vegetable origin, on their introduction into the animal body, stimulated the formation of neutralizing bodies, which were termed antibodies, there has been constant investigation of the laws governing such neutralizations. Nevertheless, in spite of the enormous amount of work that has been accomplished in this field, the true nature of neither antigen nor antibody has been definitely established.

The present series of papers is concerned with attempts to secure purified solutions of antibody, which would allow direct methods of examination as to their nature and which could be used therapeutically without the introduction of extraneous material, having a dangerous or disagreeable effect.

The purification of antibody, as it has been attempted, may be accomplished by the direct and indirect methods.

The direct methods involve precipitation of the antibody together with certain of the other serum constituents and the redissolving of such precipitates. Such procedures must necessarily fail to give pure solutions.

¹ Presented in abstract form November, 1919—Pathological Society of Philadelphia—Reported in Proceedings of Pathological Society of Philadelphia. 1920, Volume XL Old Series, Volume XXII New Series.

The indirect methods may be classified as:

1. Direct adsorption methods.
2. Indirect adsorption methods.
3. Absorption methods.

All such indirect methods involve the use of some formed material with which the antibody combines; after the combination has been washed free of most of the other serum constituents the antibody is then removed from its attachment.

Since the present work is concerned with the indirect method, only this phase will be reviewed.

THE USE OF INERT ADSORPTION MATERIALS

It has been shown by several observers, Andrejew (1), Landsteiner and Reich (2), Jaque and Kunz (3), that animal charcoal, kaolin and other substances take up antibody from serum solutions, together with certain of the other constituents, and that freeing of the antibody from this combination is excessively difficult in the test tube, but that this may occur in the animal body. Ssbolew (4), using iron hydroxide with well diluted immune serum, obtained a precipitate which contained practically all the antibody content. Emulsions of this precipitate, when injected into the animal body, acted as antibody to subsequent injections of antigen, demonstrating that dissociation of the combination had taken place with a freeing of the antibodies. However, all attempts to cause a similar dissociation in the test tube failed.

INDIRECT ADSORPTION

Gay (5) (6), Zinsser (7), Muir and Martin (8) found that when a serum containing the precipitin antibody was brought in contact with an appropriate precipitinogen, the resulting precipitate carried down with it a large proportion of all the antibodies contained in the serum.

Gay and Chickering (9) and Chickering (10), making use of this phenomenon, purified antipneumococcus serum; Weinstein (11) purified an antityphoid serum and obtained antibody

solutions by dissolving such precipitates in weak alkalis. Although such solutions contained antibody, they also contained a definite amount of the serum proteins, since the precipitate in such cases is, at least in part, derived from the serum.

ABSORPTION METHODS. REVERSIBILITY OF THE ANTIBODY- ANTIGEN REACTION

It was early recognized that antibody would combine with its homologous antigen and in the case of formed antigens such a combination could be washed free of most of the other serum constituents. Many attempts were made to reverse this reaction and so obtain antibody in a more or less free state.

Aside from the obtaining of serum-free antibody, the question of the true reversibility of the reaction deserves attention and this will be taken up at a later point.

Landsteiner (12) and Landsteiner and Jagic (13) washed red blood cells agglutinated by abrin in many changes of salt solution and then by treatment with salt solution at temperature of 42°C. to 45°C. they were able to demonstrate that a certain amount of the abrin was split off from the combination, and rendered reavailable. They also showed this to be true for normal hemagglutinins. Finally, they demonstrated that continued washing with salt solution removed some bacterial agglutinins from agglutinated antigen, but that more were removed when such combinations were heated to 55°C. Although they had shown that with the normal hemagglutinins, more were rendered free by heating at 45°C. than at higher temperatures, they offer no explanation of the use of 55°C. for the dissociation of the bacterial agglutinin-agglutinin complex.

Morgenroth (14) noticed that when red blood cells, sensitized with hemolytic amboceptor, were brought in contact with unsensitized cells, some of the amboceptor was transferred to the fresh cells.

Bail and Tsuda (15) working with the cholera vibrio, injected sensitized organisms into the peritoneal cavity of guinea-pigs and after destruction of the vibrio had occurred (Pfeiffer reaction),

they reinjected the guinea-pigs with unsensitized cholera vibrios, which were in turn destroyed, but as a rule somewhat more slowly. By bleeding the guinea-pigs after such treatment, they were able to demonstrate the presence of antibody in the serum. By digestion of sensitized antigen in salt solution at 56°C., they were able to show that bactericidal antibody was reavailable but not agglutinins; a result that has been confirmed in part by our own work with dysentery antibodies.

Spaet (16), also working with cholera and cholera immune serum, digested sensitized antigen in salt solution at 42°C. and demonstrated the splitting off of bactericidal antibody. In this work, sensitization took place at 42°C. and dissociation at the same temperature. Spaet found that more dissociation occurred when inactivated serum was used for sensitization than when active serum was used, and that when the antibody solutions were reheated to 56°C., this temperature did not cause a reduction of the titer but 66°C. caused a marked reduction.

Hahn and Trommsdorf (17) treated agglutinated bacteria with N/100 sulphuric acid and regained active agglutinins.

Von Liebermann and Fenyvessy (18), employing rabbit's immune serum against pig's corpuscles, digested sensitized antigen with N/100 HCl in salt solution. These extracts were precipitated with alkali, the precipitate dissolved and purified with ether. The final solution contained both agglutinins and hemolysins but showed no albumin with the most delicate tests used.

Kosaki (19), using rabbit serum containing immune hemolytic amboceptor against sheep's red blood cells, has contributed an important paper to this subject. Finding that dissociation could be brought about in solutions of sugar containing no electrolyte, he attempted to work out the laws governing the dissociation—such as the influence of temperature and volume. The claims that he puts forth in his conclusions are not substantiated in all respects by the experimental evidence presented. His suggestion that electrolyte is necessary to the combination of antigen and antibody is a generalization that has not been proved.

Also his claim to have recovered five-sixths of the antibody

combined with the antigen, while shown in the curve tables presented, cannot be substantiated by an analysis of the experiments shown in detail. On the basis of this claim, Kosaki thinks that all such combined antibody can be recovered; but such an assumption is open to doubt.

In all of the experiments of this nature, which are shown in the literature in sufficient detail to be carefully analysed, the following fact is apparent:

An excess of sensitizing serum has been always employed.

Since it is well known that an antigen will combine with not only sufficient antibody to cause a complete reaction (such as agglutination and hemolysis) but with many times this amount, and since, in no instance has it been shown that all the combined antibody has been recovered, it follows that a true reversal of the essential antibody-antigen reaction has not been demonstrated.

It is entirely conceivable in this connection that two distinct reactions are brought in play; one, the essential reaction, taking place between the antigen and the minimal amount of antibody necessary for complete sensitization and resulting presumably in a firm combination; the other, taking place between the antigen and an additional portion of antibody, a portion that is in excess of the minimal sensitizing quantity. The combination resulting from this reaction may or may not be a firm one.

The efforts to determine whether a complete reversal of antigen-antibody reaction is possible have been made with the use of minimal sensitizing quantities and also with greater quantities of the antibodies.

Considerable technical difficulties are presented in the use of a single sensitizing dose. We may state here, however, that several attempts, which we have made, both with hemolytic amboceptor-red cell combinations and with agglutinin-agglutino-gen combinations have all failed to demonstrate any dissociation.

The work of others on dissociation of antibody in excess from its combination with antigen and, as will be seen in the following section, our own work show that only a certain proportion of attached antibody can be removed and that the amount remaining attached after such dissociation is, in all cases, somewhat

more than the minimal sensitizing dose. Thus, the assumption seems less reasonable that the antibodies are attached to antigen in two distinct portions in sharply different degrees of firmness than that a graduated variability in the firmness of the union exists, and that those antibodies which are less firmly bound are the first to be dissociated.

Although complete reversal of the antigen-antibody reaction may be impossible, there is abundant proof of the possibility of an incomplete or partial reversal enabling the production of antibody solutions in a more or less pure state.

The problem that we have undertaken was approached through the following subdivisions:

1. Improvements in the technic of obtaining antibody solutions free from other serum constituents.

2. Determination of the laws governing the dissociation of antibody-antigen combinations.

3. Study of differences in the dissociability of various antibodies from sensitized antigen.

4. Study of the chemical nature of antibody.

These question are taken up experimentally in the following sections.

ANTIBODY STUDIES

II. THE RECOVERY OF ANTIBODY FROM SENSITIZED ANTIGENS: TECHNIC

F. M. HUNTOON AND S. ETRIS

From the Mulford Biological Laboratories

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The observations of many authors, as discussed in section 1, have demonstrated that a reversal of the antigen-antibody reaction is possible, at least in part, both in *vivo* and in *vitro*.

As has been stated in the preceding section, Kosakai (19) introduced the use of solutions of various sugars for the separation of hemolytic amboceptor from sensitized corpuscles and we first determined whether this technic could be applied to other antibodies. Since agglutinins are more easily handled than other antibodies and give fairly sharp-cut results, the preliminary work was done with these antibodies.

SEPARATION OF AGGLUTININS FROM SENSITIZED BACTERIA

In order that the steps in the process of dissociation may be followed and owing to the importance of seemingly slight variations in technic, it will be necessary to give the methods employed in detail, since it has been found that antibodies vary as to the ease with which they are dissociated and fall into classes in regard to the effect of different salts on such separation.

The use of saccharose solutions in the dissociation of the agglutinin-agglutinin complex

In protocol 1, is shown an experiment demonstrating that the meningococcus agglutinin-agglutinin complex can be broken up to a certain extent in a solution of saccharose.

Protocol 1. Dissociation of meningococcus agglutinin by means of a solution of saccharose

Antigen. M137 (Gordon I); dried in vacuo. Emulsion, 0.7 gram in 300 cc. of salt solution.¹

Serum. Antimeningococcus horse serum (polyvalent).

Sensitization. A. Antigen 150 cc. + 75 cc. serum + 75 cc. salt solution.

B. Antigen 150 cc. + 30 cc. serum + 120 cc. salt solution.

These mixtures were placed in a water bath at 37°C. for one hour and during this time they were frequently shaken. They were then centrifuged and the supernatant fluids were saved for testing, under the designation "absorbed serum."

Washing of sensitized antigen. The centrifuged sediments were emulsified, each in 200 cc. of salt solution; these emulsions were centrifuged and the supernatant fluids were saved for testing under the designation "wash fluid."

Dissociation. The washed sediments, each emulsified in 150 cc. of 10 per cent saccharose solution in distilled water, were both placed in a water bath at 60°C. for one hour and then on ice over night. After centrifugation the supernatant fluids (A and B) were removed for testing under the designation "extract."

Agglutination tests of the various fluids against a meningococcus antigen (Gordon I)

TEST FLUIDS	DILUTIONS									
	C	2	5	10	25	50	100	200	400	800
Original serum.....	—						4	4	4 ₂	2
Absorbed serum A.....	—			4	4	4	2	—		
Absorbed serum B.....	—			4	2	—	—	—		
Wash fluid A.....	—	—	—	—						
Wash fluid B.....	—	—	—	—						
Extract A.....	—	4	4	4	4	4	2	—		
Extract B.....	—	4	4	4	1	—	—	—		
Saccharose solution, control.....	—	—	—	—						

Note. Numerals 1, 2, 3, 4, represent degrees of agglutination; 3 and 4 were accepted as end points.

Summary and discussion of results. The results obtained are expressed as agglutinating units in the following table.

¹ When salt solution is mentioned in the tables without qualifications physiological salt solution of 0.85 per cent is indicated.

TEST FLUIDS	VOLUME	SERUM PRESENT	TITER OF SERUM	TITER OF FLUIDS	AGGLUTI- NATING UNITS PRESENT (TITER TIMES VOL- UME)
	cc.	cc.			
Original serum.....			400		
A, sensitization.....	300	75	400	—	30,000
B, sensitization.....	300	30	400	—	12,000
A, absorbed serum.....	300	—	—	50	15,000
B, absorbed serum.....	300	—	—	10	3,000
A, extract.....	150	—	—	50	7,500
B, extract.....	150	—	—	10	1,500

It is seen that the units combined with antigen during sensitization; that is, the original number present minus the number present in the absorbed serum, were in mixture A 15,000, in mixture B 9000.

The units dissociated in the A extract were 7500 or 50 per cent of combined units; those dissociated in the B extract were 1500 or 16.6 per cent of combined units. *Hence, the number of recovered antibodies varies both in amount and percentage with the amount of serum employed for sensitization.*

The question suggested itself whether other non-electrolyte media would act in the same manner as sugar solutions. The simplest medium of this kind is distilled water.

In protocol 2, is presented an experiment in which distilled water was used as an extraction fluid.

Protocol 2. Showing the effect of the employment of distilled water as a dissociation agent with sensitized meningococci

Serum. 77179; antimeningococcus polyvalent horse serum.

Antigen. 1032 M (Gordon I); 4000 million to the cubic centimeter (heated to 65°C. for thirty minutes).

Sensitization. 5 cc. of antigen were added to 5 cc. of serum dilution (1:2½) and the mixture was placed at 37°C. for one hour; this was centrifuged and the supernatant fluid designated as absorbed serum. The sediment was then washed once with 40 cc. of salt solution and re-centrifuged and the sediment washed once with 40 cc. of distilled water.

Dissociation. The washed sediment was emulsified in 10 cc. of distilled water, and heated to 55°C. for thirty minutes. After centrifuga-

tion the supernatant fluid was designated as "extraction fluid." Agglutination tests were then carried out with the homologous antigen.

Results of test

TEST FLUIDS	DILUTIONS									
	C	2	5	10	25	50	100	200	400	800
Original serum.....	—					4	4	4	3	1
Absorbed serum.....	—			4	3	1	—	—	—	—
Extraction fluid.....	—	4	4	2	—	—	—	—	—	—

Recapitulation

Agglutinating units present in the original serum..... 400 per cc.
 Agglutinating units present in the absorption mixture... 800 (400×2)
 Agglutinating units present in the absorbed serum..... 250 (25×10)
 Agglutinating units combined..... 550
 Agglutinating units dissociated..... 50 (5×10)

The failure to recover as large a percentage of antibodies as in the previous experiment may be attributed to the fact that the antigen employed in this experiment was not as heavy as in the first and that the agglutinating units present were much less numerous.

In protocol 2, it is shown that bacillary agglutinins can be obtained with distilled water in the same manner as with saccharose solution. The presence of salt prevents in large measure such dissociation (protocol 3).

Protocol 3. Comparison of the dissociation of bacillary agglutinins in a menstruum of salt solution and of distilled water

Serum employed. Polyvalent dysentery horse serum 2998.

Antigen employed. Emulsion of dysentery bacilli (Flexner type) four billion to the cubic centimeter.

Sensitization. Two lots were prepared.

A. 15 cc. antigen + 5 cc. serum.

B. 15 cc. antigen + 5 cc. serum.

Both lots were placed in a water bath at 40°C. for one hour; these emulsions were then centrifuged and the supernatant fluids removed and saved for testing as "absorbed serum."

Washing. The centrifuged sediments were re-emulsified in 40 cc. salt solution, and recentrifuged, after which the supernatant fluids

were removed and discarded. The centrifuged sediments were then re-emulsified in 40 cc. distilled water, and recentrifuged. Finally the supernatant fluids were removed and saved for testing, being designated as "wash fluid."

Dissociation. The washed sediments were treated as follows: Lot A. re-emulsified in 10 cc. of salt solution. Lot B, re-emulsified in 10 cc. of distilled water. Both lots were then placed in a water bath at 55°C. for thirty minutes. After which they were centrifuged and the supernatant fluid from (A) was designated as "salt solution extract" and that from (B) as "distilled water extract."

Agglutination tests. Serum, wash fluid, and extracts versus dysentery antigen (Flexner type)

TEST FLUIDS	DILUTIONS										
	C	2	5	10	25	50	100	200	400	800	1600
Original serum.....	—						4	4	4	4	4
Absorbed serum.....	—				4	4	4	3	—	—	—
Wash fluid.....	—	4	—	—	—	—	—	—			
Salt solution extract.....	—	4	4	3	—	—	—	—			
Distilled water extract.....	—	4	4	4	4	4	3	1			

Summary. Original serum—5 cc contained (1600×5) 8000 agglutinating units.

Absorbed serum—20 cc. contained (200×20) 4000 agglutinating units.

Wash fluid—40 cc. contained (2×40) 80 agglutinating units.

Salt solution extract—10 cc. contained (10×10) 100 agglutinating units.

Distilled water extract—10 cc. contained (100×10) 1000 agglutinating units.

Twenty-five per cent of the combined antibody was extracted by distilled water.

Two and one-half per cent extraction effected by salt solution.

A further experiment bearing on the effect of sodium chlorid on the combination of agglutinin and agglutigen is shown in protocol 4. In this experiment a saccharose extraction fluid containing meningococcus agglutinins was employed. This solution was somewhat opalescent on account of extraction of bacterial material.

Protocol 4. Influence of sodium chloride on agglutinins in dissociation fluids

Material. An opalescent saccharose extraction fluid containing meningococcus agglutinins and bacterial extracted substance. (Original titer 1-50.)

This material was divided into two portions, A and B; of which A was used as a control and B was mixed with the amount of NaCl required to produce a final content of 0.85 per cent. Both lots were then placed at 55°C. for one hour. During this time the opalescent material in B flocculated and sedimented.

Lots A and B were centrifuged and the supernatant fluid from B was removed for testing and marked (1). The sediment from B was next emulsified in distilled water and this emulsion was centrifuged, the supernatant fluid being removed for testing and marked (2).

The washed sediment was then emulsified in 10 per cent saccharose solution in distilled water, in amount equal to the original amount B.

A and B were then placed at 55°C. for thirty minutes; after centrifugation the supernatant fluids were removed for testing, that from B being marked (3).

Agglutination tests—employing homologous antigen

TEST FLUIDS	DILUTIONS						
	C	2	5	10	25	50	100
A, control.....	—	4	4	4	1	—	—
B, supernatant (1).....	—	—	—	—			
Supernatant (2).....	—	—	—	—			
Supernatant (3).....	—	4	4	4	4	1	—

The results in protocol 4 demonstrate that at least some of the bacterial substance held in colloidal suspension in the original extraction fluid was capable of acting as agglutinin under the proper conditions, and that in fact some of the agglutinins in the original fluid were in such combination as to be no longer available since the final extraction fluid shows a higher titer than the original.

Also the question is raised if any of the agglutinins tested under these conditions could be said to be in a strictly free state, since it is conceivable that agglutinins held in combina-

tion by antigen that has been more or less injured by manipulation will become transferred to fresh antigen and produce their effects.

Morgenroth (14) has shown that highly sensitized red cells will give up part of the amboceptor to fresh red blood cells and it is possible that a similar process has taken place in this instance. The following protocol 5 illustrates the effect of reheating at 55°C. and higher temperatures on the number of agglutinins extracted.

Protocol 5. Effect of heat on extraction (upper limits)

Serum. Polyvalent antimeningococcus (horse).

Antigen. 137 (Gordon I); vacuum dried; 0.5 gram in 160 cc. salt solution.

Sensitization. Ten lots prepared for sensitization. 15 cc. of serum was mixed with 15 cc. of antigen (showed immediate agglutination) and placed at 55°C. for 30 minutes with frequent shaking. After centrifugation the supernatant fluids were mixed and saved under the designation, "absorbed serum."

Washing. Each of the sediments was emulsified in 30 cc. of distilled water and then recentrifuged. The supernatant fluid was removed and labelled "wash fluid."

Dissociation. To the washed sediments were added 20 cc. of a 10 per cent saccharose solution in distilled water. After emulsifying the sediments, they were placed at 55°C. for thirty minutes and then on ice over night.

The emulsions were mixed and divided into 5 lots.

Treatment. Lot 1. Centrifuged and the supernatant fluid saved.

Lot 2. Reheated to 55°C. thirty minutes, centrifuged and the supernatant fluid saved.

Lot 3. Reheated to 60°C. thirty minutes, centrifuged and the supernatant fluid saved.

Lot 4. Reheated to 65°C. thirty minutes, centrifuged and the supernatant fluid saved.

Lot 5. Reheated to 70°C. thirty minutes, centrifuged and the supernatant fluid saved.

Agglutination tests were then performed, employing the above fluids and fresh 137 meningococcus antigen.

TEST FLUIDS			DILUTIONS							
			C	2	5	25	50	100	200	400
Original serum			—	4	4	4	4	4	4	1
Absorbed serum			—	4	4	4	4	1	—	—
Distilled water washings			—	—	—	—	—	—	—	—
EXTRACT	FIRST HEATING	SECOND HEATING								
1	55°C.	0 supernatant	—	4	4	4	3	1	—	—
2	55°C.	55°C. supernatant	—	4	4	4	3	1	—	—
3	55°C.	60°C. supernatant	—	4	4	4	3	1	—	—
4	55°C.	65°C. supernatant	—	4	4	3	1	—	—	—
5	55°C.	70°C. supernatant	—	2	1	—	—	—	—	—

Sediment of lot 1 was reheated to 55°C. in the presence of 30 cc. of fresh saccharose solution, centrifuged, and an agglutination test carried out with this re-extraction fluid. The result is shown below.

	DILUTIONS				
	C	2	5	16	25
Centrifuged—supernatant extract	—	4	4	1	—

Summary by agglutinating units. (Units = titer \times volume.)

Present in original absorption $15 \times 200 = 3000$ units.

Present in absorbed serum $30 \times 50 = 1500$ units.

Present in extracts (1) (2) (3) $20 \times 50 = 1000$ units.

Present in extract (4) $20 \times 25 = 500$ units.

Present in extract (5) $20 \times 2 = 40$ units.

Present in re-extract of sediment (1) $30 \times 5 = 150$ units.

This experiment shows that reheating at 55°C. or 60°C. does not increase the number of agglutinins extracted, that 65°C. reduces the number, and 70°C. almost eliminates the agglutinin. The results show that it is unnecessary to employ temperatures above 55°C. but that the addition of fresh saccharose solution to the sediment and reheating splits off more agglutinin.

Portions of the extraction fluids showing agglutinin content were mixed and employed for chemical tests to determine the nature of the protein present, if any.

All of the usual protein tests were negative with the exception of the ninhydrin reaction, showing that no coagulable protein or demonstrable monoamino acids were present. The nitrogen content was found to be 0.16 mgm. per cubic centimeter.

The agglutination tests indicate that in the original absorption mixture 3000 units were present and in the extraction fluids 1000 units or 33 per cent of the original. If we assume that the original serum contains 13 mgm. of nitrogen per cubic centimeter, the original absorption contained 13×15 or 195 mgm. of serum nitrogen, the final extraction contained 0.16×20 or 3.2 mgm. which equals 1.6 per cent of the original serum. That is, the reduction in agglutinating power is 66 per cent and the reduction in nitrogen content, 98.4 per cent.

The discussion of the chemical results will be taken up more in detail in a subsequent section.

Since it was desirable to remove the sugar from the extraction fluids, experiments in "salting out" and dialysis were undertaken. In protocols 6a and 6b are presented details of two experiments showing the effect of the addition of ammonium sulphate to the extraction fluids, and the re-extraction of the precipitates so obtained.

Protocol 6. (a) Effect of the addition of ammonium sulphate to an extraction fluid containing meningococcus agglutinins

Extraction fluid. Ten per cent saccharose in distilled water. The extraction fluid was divided into 5 portions of 4.5 cc. each which were treated as follows:

1. Untreated.
2. Added 2 small crystals of ammonium sulphate. Result—slight flocculation.
3. Added 1 gram of ammonium sulphate. Result—precipitation.
4. Added 2 grams of ammonium sulphate. Result—precipitation.
5. Added 3 grams of ammonium sulphate. Result—precipitation.

The water clear supernatant fluids, obtained from the above portions by centrifugation were tested for agglutinin content.

Results of agglutination tests

SUPERNATANT FLUIDS	DILUTIONS					
	C	2	5	10	25	50
(1).....	—	4	4	4	4	—
(2).....	—	4	4	3	—	—
(3).....	—	—	—	—	—	—
(4).....	—	—	—	—	—	—
(5).....	—	—	—	—	—	—

It is seen that small amounts of ammonium sulphate caused a precipitation of the bacterial substance, which was held in colloidal suspension, but such precipitation was accompanied by a loss of antibody content which became complete when larger amounts of ammonium sulphate were used.

b. Dissociation by means of distilled water, distilled water plus HCl and salt solution plus HCl, of the precipitates derived by the addition of ammonium sulphate to extraction fluids (10 per cent saccharose) before and after the removal of the mass of bacteria by centrifugation.

Meningococci, sensitized and washed free of serum were emulsified in 10 per cent saccharose solution and the emulsion was then heated to 55°C. for thirty minutes.

The emulsion was then divided into ten 20 cc. portions. An extra portion was centrifuged and the supernatant fluid retained as a control.

Five portions remained uncentrifuged. Marked A.

Five portions centrifuged; the supernatant fluids removed. Marked B.

To each portion of A and B were added 0.15 cc. of a 1 per cent solution of ammonium sulphate and the mixtures were placed in the water bath at 55°C. for thirty minutes.

Flocculation occurred in all the mixtures.

These were then centrifuged; the clear supernatant fluids were removed and tested.

Agglutination tests of supernatant fluids

TEST FLUIDS	DILUTIONS					
	C	2	5	10	25	50
Original extract.....	—	4	4	4	4	4
Supernatant A.....	—	—	—	—		
Supernatant B.....	—	—	—	—		

It is seen that the agglutinins present in the original extract have apparently been precipitated by means of the ammonium sulphate additions and should be found with the precipitates.

The sediments from portions A and B were emulsified in the following solutions.

Sediments A.

1. 8 cc. distilled water.
2. 7.6 cc. distilled water + 0.4 cc. $N/1$ HCl = $N/20$ HCl.
3. 7.8 cc. distilled water + 0.2 cc. $N/1$ HCl = $N/40$ HCl.
4. 7.6 cc. salt solution + 0.4 cc. $N/1$ HCl = $N/20$ HCl.
5. 7.8 cc. salt solution + 0.2 cc. $N/1$ HCl = $N/40$ HCl.

Sediments B.

1. 8 cc. distilled water.
2. 7.6 cc. distilled water + 0.4 cc. $N/1$ HCl = $N/20$ HCl.
3. 7.8 cc. distilled water + 0.2 cc. $N/1$ HCl = $N/40$ HCl.
4. 7.6 cc. salt solution + 0.4 cc. $N/1$ HCl = $N/20$ HCl.
5. 7.8 cc. salt solution + 0.2 cc. $N/1$ HCl = $N/40$ HCl.

The emulsions were then placed at 55°C . for fifteen minutes, and centrifuged. The slightly opalescent supernatants were removed. The acid extracts were neutralized with NaOH and became water clear. All extracts were next tested against fresh antigen at dilution $1/2$ and all showed immediate agglutination. Some, at least, of the agglutinins therefore were present with the precipitate and were dissociated by the solutions employed.

Agglutination tests of final supernatant fluids

TEST FLUIDS	DILUTIONS				
	2	4	10	25	50
Original extract.....	4	4	4	4	1
A. 1. Supernatant.....	4	4	1	—	—
2. Supernatant.....	4	4	1	—	—
3. Supernatant.....	4	4	1	—	—
B. 1. Supernatant.....	4	4	1	—	—
2. Supernatant.....	4	4	1	—	—
3. Supernatant.....	4	4	1	—	—

Note. The remainder of the supernatant fluids were lost through an accident.

It will be noted that the results of this experiment indicate:

1. That it is possible by "salting out" methods to obtain solutions of antibody practically free from sugar.

2. That the addition of acid shows no advantage in the final dissociation over the use of distilled water alone.

3. That only 6 per cent of the original antibody content was recovered.

The results of these tests show that it is possible by the use of the salting out method to obtain solutions free from most of the sugar, but that such methods are only feasible on an experimental scale owing to the great loss of the contained antibody. Dialysis of agglutinin extracts was not attempted, but will be considered under extraction of protective antibody.

SEPARATION OF BACTERICIDAL ANTIBODIES FROM SENSITIZED BACTERIA

In protocol 7 is shown an experiment with a bactericidal dysentery serum.

Protocol 7. Dissociation of bactericidal antibodies from sensitized bacteria

Serum. Antidysentery polyvalent horse serum.

Extract. Distilled water extract from 7 A dysentery (protocol 3).

Antigen. Living dysentery bacilli (Flexner type).

Two series were set up as follows:

(I)

A. 1 cc. antigen, plus 0.1 cc. dysentery serum, plus 0.9 cc. salt solution.

B. 1 cc. antigen, plus 0.1 cc. dysentery serum, plus 0.1 cc. complement (1:3) plus 0.8 cc. salt solution.

C. 1 cc. antigen, plus 0.5 cc. distilled water extract, plus 0.1 cc. complement (1:3) plus 0.4 cc. salt solution.

These mixtures were placed at 37°C. for one hour, and then culture plates were made in duplicate using 0.001 cc. from each mixture. After incubation, these plates showed the following counts:

A. Control—270 colonies = 270,000 original strength.

B. Serum control—110 colonies = 110,000; loss 160,000.

C. Extract—26 colonies = 26,000; loss 244,000.

Accordingly, the extract, volume for volume, killed approximately 42 per cent of the number of dysentery bacilli killed by the serum.

(II)

Duplication of the above experiment made two days later.

A. Control—1 cc. antigen, plus 0.2 cc. immune serum, plus 0.6 cc. salt solution.

B. Serum control—1 cc. antigen plus 0.2 cc. immune serum plus 0.3 cc. complement (1:5) plus 0.3 cc. salt solution.

C. Extract—1 cc. antigen plus 0.5 cc. extract plus 0.3 cc. complement (1:5).

Placed at 37°C. for one hour.

Duplicate plates made from 0.001 cc. and 0.0001 cc.

A. Control—0.001 cc. —colonies uncountable. 0.0001 cc.—average 54 = 540,000 per cc.

B. Serum control 0.001 cc.—72 = 72,000, loss 468,000.

C. Extract—0.001 cc.—63 = 63,000, loss 477,000.

Hence the extract has approximately 36 per cent of the value of the original serum.

This shows that distilled water splits off a very considerable portion of the combined bactericidal bodies and renders them reavailable.

In this connection arises the question of the relation of sodium chlorid to the dissociation of this antibody, and an attempt at an answer is made in the following protocol.

Protocol 8. Comparison of dissociation of agglutinin and bactericidal antibody in distilled water and salt solution (influence of sodium chlorid on dissociation)

Serum. Antidysentery polyvalent horse serum.

Antigen. Emulsion of dysentery bacilli (Flexner) 5000 million bacilli to the cubic centimeter.

Sensitization. Two sets were prepared:

A. 10 cc. emulsion + 5 cc. serum.

B. 10 cc. emulsion + 5 cc. serum.

Both sets were placed at 37°C. for one hour, and then centrifuged.

Washing. The centrifuged sediments were emulsified in 30 cc. of ice cold salt solution, and then recentrifuged. This process was repeated.

Dissociation. The twice washed sediments of the two sets were emulsified as follow:

A. In 10 cc. of salt solution.

B. In 10 cc. of distilled water.

Both sets were placed at 55°C. for thirty minutes, centrifuged, and the supernatant fluids removed and marked,—A. Salt solution extract; B. Distilled water extract.

Agglutination tests were carried out employing the extracts versus dysentery antigen.

EXTRACTS	DILUTIONS					
	2	5	10	25	50	100
A. Salt solution	4	3	1	—	—	—
B. Distilled water	4	4	4	4	3	—

Hence distilled water dissociates ten times as many agglutinins as salt solution.

Bactericidal tests were also made by employing these extracts versus dysentery antigen.

EXTRACTS	DYSENTERY EMULSION	SALT SOLUTION	COMPLEMENT (DILUTION 1:5)
	cc.	cc.	cc.
(1) A. 0.5 cc.	1.0	—	0.3
(2) B. 0.5 cc.	1.0	—	0.3
(3) A. 0.5 cc. (control)	1.0	0.3	—

These sets were placed at 37°C. for one hour, and 0.0001 cc. from each lot was plated (four duplicate plates).

Average of plate count: (1) A = 35, (2) B = 12, (3) A (control) = 53.

Summary. Control = 530,000 organisms per cubic centimeter.

A. Salt solution extract = 350,000 organisms per cubic centimeter.

B. Distilled water extract = 120,000 organisms per cubic centimeter.

Accordingly, the salt solution extract shows the presence of 43.9 per cent as many bactericidal antibodies as the distilled water extract.

Although a difference is noted between the amount of bactericidal antibody removed in salt solution and in distilled water, the difference is not as marked as that seen with agglutinin (see protocol 7).

SEPARATION OF THE PROTECTIVE ANTIBODY FROM SENSITIZED PNEUMOCOCCI

The application of various methods for accomplishing such extraction is shown in the following protocols (9 to 27).

The ultimate aim held in view for such an investigation was the obtaining of protective antibodies in solutions free from serum proteins and of sufficient concentration to be available for therapeutic use; also in a menstruum, preferably salt solution, which would be innocuous.

The technic of obtaining such antibody solutions as were desired, containing an adequate concentration of antibody and a minimum of serum proteins, may be divided into three main steps, modifications of which have a very important bearing on the final result.

1. Methods of sensitization of the antigen.
2. Methods of washing the serum from the sensitized bacteria.
3. Methods of dissociation of the antibody-antigen combination.

Since the dissociation methods are fundamental, this phase of the question was first investigated.

In the following protocol (9) saccharose solution was employed as an extraction agent.

Protocol 9. Saccharose extraction of protective antibody from sensitized pneumococci

Serum. Antipneumococcus, polyvalent horse serum 4018.

Antigen. Heavy emulsion of living pneumococci, mixed types I, II, III.

Sensitization. 1. Control, 5 cc. of serum added to 5 cc. of salt solution.

2. 20 cc. of serum added to 20 cc. of antigen.

These were placed at 37°C. for one hour, and then centrifuged. The supernatant fluid of (2) was saved and designated as "absorbed serum."

Washing. The sediment from (2) was emulsified in 40 cc. of salt solution, recentrifuged and the supernatant fluid discarded.

Dissociation. The washed sediment was emulsified in 10 cc. of a 10 per cent saccharose solution in distilled water.

The control (1) and the emulsion (2) were heated to 55°C. for thirty minutes. Both were centrifuged and the slightly opalescent supernatant fluid of (2) removed for testing.

Protection tests on mice with a virulent type I pneumococcus

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULT
	cc.		cc.	
Control serum diluted to equal dilution of absorbed serum. . . .	0.4	Pneumococcus type I	0.01	S S
	0.4	Pneumococcus type I	0.1	S S
	0.4	Pneumococcus type I	0.2	S S
Absorbed serum.	0.4	Pneumococcus type I	0.01	S 70
	0.4	Pneumococcus type I	0.1	S 48
	0.4	Pneumococcus type I	0.2	70 70
Saccharose extraction fluid—volume one-half that of original serum.	0.4	Pneumococcus type I	0.0001	S S
	0.4	Pneumococcus type I	0.001	S S
	0.4	Pneumococcus type I	0.01	S S
None. Virulence control.		Pneumococcus type I	0.000000001	48 48

Note. In this and subsequent protocols showing protection tests, unless specified otherwise, the injection of protective material and culture is made intraperitoneally in separate doses but within 1 minute.

"S" in protocols indicates survivals for 96 hours.

Numerals indicate hour of death.

These tests demonstrate that a considerable number of protective antibodies have been removed from the serum employed, and that a definite number appear in the extraction fluid.

A second experiment is shown in protocol 10 in which a different serum is employed together with type 1 antigen only and in which an additional washing with distilled water was introduced.

Protocol 10. Extraction of antibody from sensitized pneumococcus antigen by means of a 10 per cent solution of saccharose in distilled water

Serum. 79306; polyvalent antipneumococcus horse serum.

Antigen. Heavy emulsion of type I pneumococcus (living).

Sensitization. 50 cc. serum added to 50 cc. antigen. The mixture was placed at 37°C. for two hours and then centrifuged.

Washing. The centrifuged sensitized sediment was emulsified in 100 cc. of salt solution. The emulsion was centrifuged. The sediment was again emulsified in 100 cc. of distilled water and again centrifuged.

Dissociation. The washed sediment was now emulsified in 50 cc. (equal to the original volume of serum employed in sensitization) of a 10 per cent solution of saccharose in distilled water.

The emulsion was heated at 55°C. for forty-five minutes, and centrifuged. The supernatant fluid was removed, designated as "extract" and employed to determine antibody content.

Protection tests against Pneumococci

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULT
	cc.		cc.	
Original serum	0.2	Pneumococcus type I	0.1	S S
	0.2	Pneumococcus type I	0.2	S S
	0.2	Pneumococcus type I	0.5	S S
"Extract"	0.4	Pneumococcus type I	0.01	S S
	0.4	Pneumococcus type I	0.1	S S
	0.4	Pneumococcus type I	0.2	48 48
Virulence controls . . .		Pneumococcus type I	0.00000001	S S
		Pneumococcus type I	0.00000001	55 48

A third experiment made with the same antigen as in protocol 10 but with a different serum not only confirms the results shown in protocols 9 and 10, but in addition takes up the problems of sterilization by means of filtration, and the further purification of the final product.

Protocol 11. (1) *Extraction of antibody from sensitized pneumococcus antigen.* (2) *Effect of passage through filter candles on antibody content of the extract.* (3) *Purification of the extract: (a) by "salting out" methods; (b) by dialysis*

Serum. 79307 antipneumococcus horse serum.

Antigen. A heavy emulsion of type 1 pneumococcus in salt solution.

Sensitization. 1. 900 cc. of serum were added to 900 cc. of the antigen.

2. The mixture was placed at 37°C. for two hours.

3. The mixture was centrifuged in the "Sharples" centrifuge.

Washing. 1. The packed sediment was then washed "in situ" with 2 liters of salt solution followed by 2 liters of distilled water.

Dissociation. 1. The sensitized and washed bacteria (sediment) were emulsified in 1800 cc. (twice the original volume of serum) of a 10 per cent solution of saccharose in distilled water.

2. The emulsion was heated at 55°C. to 60°C. for forty-five minutes.

3. The emulsion was then divided into two portions: A, 200 cc.; B, 1600 cc.

4. The treatment of A was as follows: Centrifuged in a cup centrifuge and the supernatant fluid removed, being designated "A (extract centrifuged)." 100 cc. of A (extract centrifuged) was next passed through a small filter candle and gave a clear solution. This filtrate was designated as "A (extract filtered)."

5. The treatment of B was as follows: Passed through a "Sharples" centrifuge and the opalescent supernatant fluid saved for further work, being designated "B (extract centrifuged)."

Protection test with A extracts against type I Pneumococcus

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULT
	cc.		cc.	
Original serum	0.2	Pneumococcus type I	0.1	S S
	0.2	Pneumococcus type I	0.2	S S
	0.2	Pneumococcus type I	0.5	S S
Absorbed serum	0.4	Pneumococcus type I	0.01	S S
	0.4	Pneumococcus type I	0.1	S S
	0.4	Pneumococcus type I	0.2	72 48
A, extract centrifuged	0.4	Pneumococcus type I	0.01	S S
	0.4	Pneumococcus type I	0.1	72 48
	0.4	Pneumococcus type I	0.2	72 48
A, extract filtered	0.4	Pneumococcus type I	0.001	S S
	0.4	Pneumococcus type I	0.01	48 48
Virulence controls		Pneumococcus type I	0.00000001	48 48
		Pneumococcus type I	0.00000001	48 48

Note reduction of antibody content by filtration.

Experiments with B extract centrifuged were made as follows: Of this extract, 20 cc. were retained for control testing, and then 1500 cc. were passed rapidly through a large filter candle under considerable

pressure. This filtration fluid showed a slight amount of opalescence; it was designated as "B (extract-filtered)."

Protection tests with B extracts against type I Pneumococcus

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULT
	cc.		cc.	
B, extract centrifuged {	0.4	Pneumococcus type I	0.01	S S
	0.4	Pneumococcus type I	0.1	S 70
	0.4	Pneumococcus type I	0.2	S S
B, extract filtered . . . {	0.4	Pneumococcus type I	0.01	S S
	0.4	Pneumococcus type I	0.1	S 48
Virulence controls . . . {		Pneumococcus type I	0.000000001	S S
		Pneumococcus type I	0.000000001	55 48

It is seen that rapid filtration does not reduce the antibody content in the same degree as slower filtration.

3 (a) "*Salting out*" of filtered extract. Two 15 cc. portions of "B extract filtered" each received an addition of 15 cc. of a saturated solution of ammonium sulphate. These mixtures were placed at room temperature for two hours, when they showed a distinct flocculation. Both portions were now centrifuged and the supernatant fluids removed, being designated as "Ammonium sulphate supernatant."

The two sediments were treated as follows: (1) Emulsified in 10 cc. of salt solution. (2) Emulsified in 10 cc. of distilled water.

Both were centrifuged, and the supernatant fluids removed, being respectively designated: (1) "Final salt solution extract." (2) "Final distilled water extract."

Protection tests of B (extract filtered) treated with ammonium sulphate and tested against type I Pneumococcus

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULT
	cc.		cc.	
"Ammonium sulphate" supernatant	0.2	Pneumococcus type I	0.001	S 70
(1) Final salt solution extract	0.4	Pneumococcus type I	0.001	S 70
(2) Final distilled water extract	0.4	Pneumococcus type I	0.001	S S
	0.4	Pneumococcus type I	0.01	S S
Virulence controls . . . {		Pneumococcus type I	0.000000001	S S
		Pneumococcus type I	0.000000001	48 55

It is seen in this experiment:

1. That not all of the antibody present is precipitated by means of half saturated ammonium sulphate.
2. That distilled water is a better solvent for the precipitate than salt solution.
3. That it is possible by the means employed to obtain antibody solutions free from all but a trace of sugar and ammonium sulphate.

3 (b). *Dialysis of antibody extract.* "B (extract filtered.)" Of "B (extract filtered)" 105 cc. were placed in a dialysing bag, a small amount of chloroform was added as a preservative and the bag was placed in running tap water.

Results of dialysis

DAYS	VOLUME	SUGAR CONTENT
	cc.	per cent
0	105	10
5	150	1.4
9	170	0

At end of nine days a flocculent precipitate was visible in the dialysing solution. The material was then removed from the dialysing bag and centrifuged, and the clear supernatant fluid was designated as "Dialysed supernatant."

The sediment (globulin?) was next shaken up in 10 cc. of salt solution and thereby brought into partial solution. The emulsion was re-centrifuged and the supernatant fluid was removed, being designated as "Dissolved sediment."

Protection tests of dialysed material against type I Pneumococci

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULT
	cc.		cc.	
'Dialysed supernatant'.....{	0.7	Pneumococcus type I	0.001	S S
	0.7	Pneumococcus type I	0.01	18 18
	0.7	Pneumococcus type I	0.1	S 18
"Dissolved sediment".....{	0.7	Pneumococcus type I	0.0001	72 18
	0.7	Pneumococcus type I	0.001	18 18
Control serum.....{	0.2	Pneumococcus type I	0.1	S S
	0.2	Pneumococcus type I	0.3	S S

Note. 0.7 cc. of dialysed supernatant is the approximate volumetric equivalent of 0.2 cc. of the original mother serum.

It will be noted:

1. That some reduction in the value of the antibody content has taken place during dialysation.
2. That the precipitate formed carried little or no antibody with it.

Summary. The series of experiments shown in protocol 11, demonstrate that, with the technic employed, the following conclusions are warranted:

1. That a sterile solution containing antibody and saccharose could be obtained, but that a reduction of the antibody content is likely to occur from filtration through filter candles.
2. That by "salting out" methods such solutions can be further purified, but with loss of antibody.

The further determination of a possible technic for the production of solutions containing antibodies, but free from all except traces of sugar and salts, was now undertaken, with results as shown in protocol 12.

Protocol 12. Purification of antibody extract. (1) Influence of the addition of salts on the protective antibody. (2) Differences between salt solution and distilled water as final extraction agents

Serum. Anti-pneumococcic horse serum.

Antigen. Heavy emulsion of type I pneumococci.

Sensitization. 2000 cc. of serum + 200 cc. of antigen. This mixture was placed at 55°C. for thirty minutes, and centrifuged in a "Sharples" centrifuge.

Washing. The packed sediment in the centrifuge was washed "*in situ*" by passing through the centrifuge 2 liters of salt solution and 2 liters of distilled water.

Dissociation. The washed sediment was emulsified in 3000 cc. of a 10 per cent solution of saccharose in distilled water. This emulsion was then heated at 55°C. for thirty minutes.

The heated sugar emulsion was divided into two portions. One portion was centrifuged in the "Sharples" and the slightly opalescent supernatant designated as "B" and the remaining uncentrifuged sugar emulsion as "A."

The A and B portions were each divided into two lots and treated as follows.

1. To one lot of each was added 0.5 per cent of its volume of a saturated solution of ammonium sulphate.

2. The mixtures were heated at 55°C. for thirty minutes.

3. They were then centrifuged and the supernatant fluids were designated as "As 1" and "Bs 1."

4. The sediments remaining were shaken up in a quantity of salt solution equal to one-third the original volume.

5. The salt solution emulsions were heated at 55°C. for thirty minutes.

6. They were then recentrifuged and the supernatant fluids were designated as "As 2" and "Bs 2."

7. The remaining sediments were shaken in distilled water of one-third the original volume.

8. Placed on ice forty-eight hours, and recentrifuged.

9. The supernatant fluids were designated as "As 3" and "Bs 3."

The various supernatants were now tested to determine the antibody content.

Protection tests

TEST FLUIDS	AMOUNT	CULTURE	AMOUNT	RESULT
	cc.		cc.	
Uncentrifuged sugar emulsion.....	0.6	Pneumococcus type I	0.01	S S
Ammonium sulphate set				
As1.....	0.6	Pneumococcus type I	0.01	24 24
As2.....	0.4	Pneumococcus type I	0.01	24 24
As3.....	0.4	Pneumococcus type I	0.01	70 70
Ammonium chloride set				
Ac1.....	0.6	Pneumococcus type I	0.01	S 24
Ac2.....	0.4	Pneumococcus type I	0.01	48 24
Ac3.....	0.4	Pneumococcus type I	0.01	S 70
Centrifuged sugar supernatant				
Ammonium sulphate set				
Bs1.....	0.6	Pneumococcus type I	0.01	S 24
Bs2.....	0.4	Pneumococcus type I	0.01	24 24
Bs3.....	0.4	Pneumococcus type I	0.01	S 70
Ammonium chloride set				
Bc1.....	0.6	Pneumococcus type I	0.01	S 24
Bc2.....	0.4	Pneumococcus type I	0.01	18 18
Bc3.....	0.4	Pneumococcus type I	0.01	S S
Virulence control.....		Pneumococcus type I	0.00000001	48 48

The distilled water final extracts were retested.

TEST FLUIDS	AMOUNT	CULTURE	AMOUNT	MICE
	cc.		cc.	
Ammonium sulphate set	0.2	Pneumococcus type I	0.001	S S
As3.....	0.2	Pneumococcus type I	0.01	S 18
	0.2	Pneumococcus type I	0.1	42 70
Ac3.....	0.2	Pneumococcus type I	0.001	S 42
	0.2	Pneumococcus type I	0.01	S 42
	0.2	Pneumococcus type I	0.1	24 70
Ammonium chloride set	0.2	Pneumococcus type I	0.001	S S
Bs3.....	0.2	Pneumococcus type I	0.01	S S
	0.2	Pneumococcus type I	0.1	70 70
Bc3.....	0.2	Pneumococcus type I	0.001	S S
	0.2	Pneumococcus type I	0.01	S S
	0.2	Pneumococcus type I	0.1	S 70
Virulence.....		Pneumococcus type I	0.00000001	48 48

The remaining lots of A and B received the addition of 0.5 per cent by volume of a saturated solution of ammonium chlorid and were treated in an exactly similar manner as above.

The corresponding supernatant fluids were designated as "Ac 1" and "Bc 1," "Ac 2" and "Bc 2," "Ac 3" and "Bc 3."

The results of the above tests indicate:

1. That ammonium chloride is a slightly better precipitating agent than ammonium sulphate.

2. That an exposure of the precipitates (salted out from extraction fluids) to distilled water at ice-box temperature for forty-eight hours gives better results than exposure to salt solution at 55°C.

3. That using the supernatant fluid of the sugar extraction fluid gives distinctly better results than using the whole emulsion.

Influence of time on distilled water extraction. Without giving the details of the experiment it may be stated here that it was found that an exposure of the final precipitate in such an experiment to distilled water for a few minutes gave no dissociation but that two hours exposure to ice box temperature was as efficient as twenty-four hours exposure at this temperature.

Influence of concentration of ammonium salts. It was found that when ten per cent by volume of a saturated ammonium sulphate solution was used as a precipitating agent, in an experiment similar to protocol 12, the number of antibodies in the final distilled water extraction was much diminished.

Influence of addition of sodium chloride on final extraction fluids. All of the final distilled water extraction fluids in protocol 12, were slightly opalescent and the attempt to render such solutions isotonic with sodium chloride resulted in a flocculation with a slight loss of antibody content.

An analysis of the results of experiment 12, apparently justifies the following conclusions in regard to the influence of the addition of small amounts of salts to the saccharose solutions of antibody, and tends to strengthen the conclusions arrived at from the study of agglutinins.

1. That little or no dissociation could be expected in the presence of sodium chloride or other salts. (This conclusion was invalidated by further work with the protective antibody.)

2. That the antibody present is not necessarily in a free state but is probably bound loosely to the bacterial substances which are held in colloidal suspension and becomes more closely bound on the addition of a salt. The latter conclusion was strengthened by many attempts at filtration through filter candles which, up to this time, always resulted in a distinct loss of antibody content.

At this point in the investigation we were led to a review of the work of Gay and Chickering (9) and of Chickering (10) on the adsorption of antibody by the precipitate, when an immune pneumococcus serum is brought in contact with a properly prepared precipitinogen. They discovered that by extraction of the washed precipitate the antibodies can be recovered, but this recovery was always accompanied by a certain percentage of demonstrable serum protein.

We confirmed their results and hoped by applying the methods at our disposal to eliminate the protein content. However, the irregularities of action with different sera and the difficulties of preparing large amounts of a suitable precipitinogen led to an abandonment of this method.

The elaborate technic which the experiment in protocol 12 seemed to require, rendered it obvious that the production of a sterile end product (if filtration was not available) could be attained only with extreme difficulty and uncertainty, and further work was undertaken with the aim of simplifying the technic and of utilizing extraction materials other than saccharose solution.

Since our evidence pointed to the influence of the absence of salts on the final dissociation, distilled water was chosen as one extraction fluid, and, since the objections to intravenous injections of dextrose are not great (in fact this substance might prove beneficial), an isotonic solution (5.6 per cent) of this sugar was also chosen.

Preliminary experiments were also undertaken to determine whether antigen treated with heat would still absorb antibody, but would, perhaps, give off the antibody in greater amounts than unaltered antigen.

An experiment giving comparative values of antigen heated to various degrees will be given later, but it is sufficient to state here that pneumococci heated to 100°C., in the presence of N/100 HCL, still combine readily with antibody. An experiment is given below, using such an antigen and showing the use of distilled water as an extraction agent.

Protocol 13. (1) The effect of boiling pneumococcus antigen in the presence of N/100 HCl on the dissociation of antibody. (2) The effect of the volume of the dissociation agent on the amount of antibody extracted. (3) The effect of repeated extraction of sensitized antigen

Antigen. Heavy emulsion of pneumococcus type 1, in salt solution, rendered acid to the strength of N/100 with HCl, heated at 100°C. for fifteen minutes in a water bath.

Serum. 70094 pneumococcus.

Sensitization. 400 cc. of serum added to 100 cc. of antigen (neutralized with NaOH neutral to bromeresol-purple). Placed at 37°C. 2 hours and on ice over night.

Washing. Centrifuged: The sediment emulsified in and washed with distilled water. Divided into two equal portions, and recentrifuged.

Dissociation. A. Added to sediment 20 cc. of distilled water (one-fifth, volume serum used).

B. Added to sediment 100 cc. of distilled water (i.e., one-half of the volume of serum used).

Both emulsions were placed at 5°C. for eighteen hours.

Centrifuged: Supernatant fluid A placed on test; supernatant fluid B placed on test.

Redissociation. To the sediment from A were added 100 cc. of distilled water and the emulsion was placed at 5°C. for 24 hours.

Centrifuged and the supernatant fluid placed on test as C.

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULT
	cc.		cc.	
A. supernatant (20 cc.) {	0.2	Pneumococcus type I	0.001	S S
	0.2	Pneumococcus type I	0.01	18 18
B. supernatant (100 cc.) {	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	52 48
Control serum.....	0.2	Pneumococcus type I	0.07	S 48
Virulence control.....			0.000000001	48 24
C. extract supernatant {	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	S 90
	0.2	Pneumococcus type I	0.01	48 48
Original mother serum { 70094.....	0.2	Pneumococcus type I	0.01	S S
	0.2	Pneumococcus type I	0.07	S 48
Virulence control.....		Pneumococcus type I	0.000000001	48 48

Note. In this and the following tables the virulent culture employed for test purposes was grown in a suitable plain broth medium and, as will be noted by a comparison of this with preceding tables, the apparent protective power of the sera is much reduced, so that readings of the results should be made in comparison with the control sera.

This experiment indicates that distilled water can be used as an extraction agent, that one exposure of the sensitized antigen to distilled water does not remove all of the attached antibody and that the volume of the exhausting agent bears no close relation to amount of extraction.

A confirmatory experiment showed the same results and also that the addition of acid to the emulsion before heating was not essential to the final result. The experiment detailed below

shows that a broth culture of pneumococcus may be used as antigen, without centrifugation, if a sufficiently heavy growth be obtained.

Protocol 14. Use of broth culture of pneumococcus (heated to 100°C.) as antigen. Influence of time on dissociation by distilled water. Relation of amount of antibody dissociated to amount retained by antigen

Antigen. Heavy growth of type 1 pneumococcus (virulent strain) in hormone broth, heated to 100°C. for ten minutes in a water bath.

Serum. 70094 antipneumococcus.

Sensitization. 10 cc. of the antigen was added to 20 cc. of serum and heated at 40°C. to 50°C. for forty-five minutes, then kept on ice eighteen hours.

Washing. The centrifuged precipitate was washed twice with 30 cc. of distilled water.

Dissociation. To the washed sediment were added 10 cc. of distilled water (half original volume of serum used). The emulsified sediment was then placed at 5°C. for five hours and 3 cc. removed for test, being designated as—"emulsion A."

The remainder was placed at 5°C. for eighteen hours, after which it was centrifuged and the supernatant fluid (opalescent) designated as "supernatant B."

The sediment was re-emulsified in 7 cc. distilled water and designated as "emulsion C."

Potency tests

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULT
	cc.		cc.	
Emulsion A (5 hour lot).....	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	S S
Supernatant B. (24 hour lot).....	0.2	Pneumococcus type I	0.001	S S
	0.2	Pneumococcus type I	0.01	18 70
	0.2	Pneumococcus type I	0.1	48 24
Emulsion C. (reemulsified) Sediment 24 hours.....	0.2	Pneumococcus type I	0.001	S S
	0.2	Pneumococcus type I	0.001	S S
	0.2	Pneumococcus type I	0.01	S 48
Virulence control.....		Pneumococcus type I	0.000000001	18 48

In this table it is evident that a considerable portion of antibody is available in the distilled water employed in making the emulsion as early as five hours; that the twenty-four hour supernatant fluid affords protection against one million fatal doses, but that as many or more antibodies remained with the sediment and are rendered available on introduction into the animal body.

The following experiment (protocol 15) was undertaken to determine the relative values of distilled water and a 5.6 per cent dextrose solution as extraction agents.

Protocol 15. (1) Comparison of distilled water and dextrose as dissociation agents. (2) Time factor in dissociation

Antigen. Broth culture: heavy growth of virulent pneumococci in hormone broth.

Serum. 70,094 antipneumococcus.

Sensitization. Four lots were prepared as follows: To 26 cc. of serum were added 13 cc. of antigen. The mixtures were placed at 50°C. for forty-five minutes; during this period they were shaken vigorously at ten minute intervals, and finally they were placed in the ice box for a period of eighteen hours.

Washing. The mixtures were centrifuged and the sediments were washed twice with distilled water.

Dissociation. Two lots. To the centrifuged and washed sediment were added 13 cc. of distilled water; that is, one-half of the original volume of serum employed for sensitization. Two lots. To the centrifuged and washed sediment were added 13 cc. of a 5.6 per cent solution of dextrose in distilled water.

The sediments were emulsified and placed at 52°C. for thirty minutes and then at 5°C. for five hours. At the end of five hours 3 cc. of each emulsion were removed and designated: Distilled water emulsion A (five-hour); dextrose emulsion B (five-hour).

The remainder of the emulsions were returned to the ice box for a period of nineteen hours. At the end of this period 3 cc. samples were again removed and designated: Distilled water emulsion C (twenty-four hour); dextrose emulsion D (twenty-four hour).

The remainder of the emulsions were centrifuged and the supernatant fluids were removed. The somewhat opalescent fluid from the distilled water lot was designated: Distilled water supernatant E (twenty-four hour).

The clear supernatant fluid from the dextrose lot was designated. Dextrose supernatant F (twenty-four hour).

The centrifuged sediments remaining were reemulsified in an equal amount of their homologous solutions and designated: Distilled water (reemulsified sediment) G; dextrose emulsion (reemulsified sediment) H.

Potency tests of extracts

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULT
	cc.		cc.	
Distilled water emulsion A (5 hour).....	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	S 70
	0.2	Pneumococcus type I	0.01	48 48
Dextrose emulsion B (5 hour).....	0.2	Pneumococcus type I	0.001	S S
	0.2	Pneumococcus type I	0.01	S S
	0.2	Pneumococcus type I	0.1	18 18
Distilled water emulsion C (24 hour)....	0.2	Pneumococcus type I	0.001	S S
	0.2	Pneumococcus type I	0.01	18 24
Dextrose emulsion D. (24 hour).....	0.2	Pneumococcus type I	0.001	S S
	0.2	Pneumococcus type I	0.01	S S
	0.2	Pneumococcus type I	0.1	18 18
Distilled water supernatant E (24 hour)..	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	24 90
Dextrose supernatant F (24 hour).....	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	48 70
Distilled water (reemulsified sediment) G.....	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	S S
	0.2	Pneumococcus type I	0.01	48 90
Dextrose emulsion (reemulsified sediment) H.....	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	S 18
	0.2	Pneumococcus type I	0.01	24 90
Original serum (70094)	0.2	Pneumococcus type I	0.01	S S
	0.2	Pneumococcus type I	0.1	40 40
Standard control serum.....	0.2	Pneumococcus type I	0.01	S S
	0.2	Pneumococcus type I	0.1	18 18
Virulence control.....		Pneumococcus type I	0.000000001	18 18

The results of the above experiment indicate:

1. That dextrose is a more efficient agent for the extraction of antibody than distilled water, both as to the time required and the amount of antibody rendered available.

2. That, although in the dextrose emulsion the number of antibodies available for protection was equal to that of the original serum, these did not appear in the supernatant fluid after centrifugation; that is, they were more or less loosely attached to the antigen in the emulsion.

The supernatant fluid from the dextrose emulsion was a perfectly clear solution and it was thought that perhaps a trace of salt in the dextrose used was responsible for both the clear solution and the failure to show the expected greater number of antibodies as compared to the distilled water extract. An analysis of the dextrose employed showed a trace of sulphates present. The significance of this was not recognized at the time, but in view of later work in which it was demonstrated that calcium sulphate prevented in part the dissociation of the protective antibody from its combination with antigen, the observation is of importance.

3. The fact that the supernatant fluid of the distilled water extraction showed a distinct opalescence while that from the dextrose extract was water-clear, although the antibody content was equal, indicates that the presence of extracted bacterial substance is not essential in order that the extraction agent may carry the antibody charge. Previous work rather indicated that this might be the case.

A repetition of this experiment with the use of a fresh serum and fresh antigen, prepared in a similar manner, was followed by failure to show any considerable number of antibodies in the dextrose extract. An investigation of the absorbed serum showed little or no loss of power as compared with the original serum. A reabsorption of this serum with another antigen yielded better results, but still not as good as those obtained in protocol 15.

It would appear that the absorption value of antigen varies and also that not all samples of serum were equally available for the purpose desired. This is illustrated in the experiment detailed in protocol 16.

Protocol 16. (1) Failure of one sensitization. (2) Re-absorption of the absorbed serum with a fresh antigen (resensitization), allowing a recovery of more antibody in the final extraction

Serum. 81089 antipneumococcus.

Antigen. Pneumococcus, twenty-four hour culture in hormone broth, heated at 100°C., fifteen minutes.

Sensitization. Of the serum, 200 cc. were added to 100 cc. of antigen and placed first at 40°C. for two hours, then at 5°C. for twenty hours. This mixture was centrifuged and the supernatant fluid saved under the designation "absorbed serum" (dilution 2 to 3 of the original).

Washing. The sediment was washed with 400 cc. of distilled water.

Dissociation. The washed sediment was emulsified in 100 cc. of 5.6 per cent dextrose solution in distilled water, and the mixture was placed at 55°C. for thirty minutes and on ice over night. A portion was removed for test—"Dextrose emulsion (1)."

Re-absorption (Sensitization). 26 cc. of the absorbed serum (see above) were added to 13 cc. of a second antigen, and the mixture was placed at 50°C. for one hour and at 20°C. for two hours. It was then centrifuged and the supernatant fluid designated as "reabsorbed serum."

Washing. The centrifuged sediment was emulsified in and washed with 40 cc. of distilled water.

Potency tests

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULTS
	cc.		cc.	
Standard control serum	0.2	Pneumococcus type I	0.01	S S
	0.2	Pneumococcus type I	0.1	S 24
Original serum 81089	0.2	Pneumococcus type I	0.01	S S
	0.2	Pneumococcus type I	0.1	S 70
Absorbed serum (original)	0.3	Pneumococcus type I	0.01	S S
	0.3	Pneumococcus type I	0.1	S 48
Reabsorbed serum	0.4	Pneumococcus type I	0.001	S S
	0.4	Pneumococcus type I	0.01	70 24
Dextrose emulsion (1) (original)	0.2	Pneumococcus type I	0.001	18 70
	0.2	Pneumococcus type I	0.01	18 24
Dextrose emulsion (2) (second lot)	0.2	Pneumococcus type I	0.001	S S
	0.2	Pneumococcus type I	0.01	48 48

Dissociation. The washed sediment was emulsified in 9 cc. of 5.6 per cent dextrose solution and heated at 55°C. for forty-five minutes, cooled and tested,—“dextrose emulsion (2).”

It is clear that in the original absorption, few, if any antibodies went into combination with the antigen employed but when the same serum was reabsorbed with another antigen grown a day later the antibodies then combined.

At this point we were led to investigate the dissociation effects of salt solution, although our evidence pointed strongly to the restraining influence that even small quantities of salt had on such a reaction. The bactericidal experiments given before had not at this time been undertaken. It was argued that, if it was not the absence of sodium chlorid as such that governed the splitting of combined antibody, such effect might be laid to differences in the tonic conditions surrounding the organisms at the time of sensitization and at the time of dissociation.

The experiments were designed so that the mixture of serum and antigen should be as hypotonic as was possible without causing a precipitation of globulin from the serum, that these conditions should remain constant during the washing and that finally the sensitized organisms should be suddenly exposed to an isotonic solution of salt and then treated as before with heat.

To our surprise we found that the salt solution extract gave practically as powerful protection as the best of the previous extracts with salt-free material; accordingly, it became necessary to divide antibodies into at least two groups as regard their sensitivity to salt solution dissociation.

Whether those antibodies, which are dissociated in salt solution, are the same as the amboceptors is very difficult to determine. The bactericidal antibody, which would lend itself to this determination, is certainly not so sensitive to the influence of salt as agglutinin; on the other hand we have never, except in one instance, been able to show that complement-fixing bodies were extractable by any method. Kosakai (19) claims and demonstrates that fewer hemolytic amboceptors are regained in salt solution than in a salt-free saccharose solution. Accord-

ingly, the evidence available fails to correlate, as regards the antibodies, the requirement of complement with the reaction of dissociation as influenced by the presence of sodium chlorid.

The adoption of sensitization in hypotonic solutions was based on the results obtained in protocol 17.

Protocol 17. Differences in amount of extractable antibody.; (A) when sensitization takes place in an isotonic solution; (b) when sensitization takes place in a hypotonic solution.

Antigen. Pneumococcus type I, virulent, one week's growth in hormone broth. Two lots (40 cc. each) transferred to centrifuge tubes and centrifuged.

A. The sediment in one tube was emulsified in 15 cc. of physiological salt solution.

B. The sediment in one tube was emulsified in 15 cc. of distilled water.

Serum. Anti-pneumococcic.

Sensitization. A. The salt solution emulsion of antigen (15 cc.) was added to (5 cc.) serum.

B. The distilled water emulsion of antigen (15 cc.) was added to (5 cc.) serum.

Both sets were heated at 40° to 45°C. for two hours.

Washing. A. The mixture was centrifuged and the sediment re-emulsified in 20 cc. of salt solution (0.85 per cent). This emulsion was recentrifuged and the sediment was again emulsified in 20 cc. of distilled water. The mixture was again centrifuged and the supernatant fluid was discarded.

B. The mixture was centrifuged and the sediment was re-emulsified in 20 cc. of salt solution (0.21 per cent). It was then recentrifuged and the sediment was again emulsified in 20 cc. of salt solution (0.21 per cent). The mixture was again centrifuged and the supernatant fluid was discarded.

Dissociation. To the washed sediments of both A and B were added 10 cc. (twice the original volume of serum) of physiological salt solution and the sediments were emulsified; both emulsions were then heated at 55°C. for thirty minutes and centrifuged and the supernatant fluids were tested for potency.

Protection tests

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULTS
	cc.		cc.	
=	0.2	Pneumococcus type I	0.00001	S 48
A. Supernatant (iso- tonic sensitization).	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	40 40
	0.2	Pneumococcus type I	0.01	18 18
B. Supernatant (hypo- tonic sensitization).	0.2	Pneumococcus type I	0.00001	S S
	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	S S
	0.2	Pneumococcus type I	0.01	40 70
Original serum	0.2	Pneumococcus type I	0.01	S 90
	0.2	Pneumococcus type I	0.05	18 70
Virulence control		Pneumococcus type I	0.0000000001	40 40
		Pneumococcus type I	0.000000001	40 40

This experiment demonstrates that better results can be obtained by sensitization in hypotonic than in isotonic solutions.

The results obtained with this experiment were repeatedly confirmed and, since this technic gave us an isotonic solution, it was adhered to for a considerable period and the extracts obtained were used in the attempt to ascertain something in regard to the nature of the material contained in such extracts. The chemical work on this point will be taken up in the following section.

We may state here that all the usual reactions for serum protein failed to give a positive result, although a measurable quantity of nitrogenous material was present. The material in these extracts could presumably be antibody, or a substance associated with antibody derived from the serum, or bacterial extracted substances—either that portion which acts as antigen or merely fortuitous portions.

Attempts were also made to determine the presence of serum proteins by sensitization methods which will be given in detail in the following section.

The main points which have been demonstrated in the preceding pages may be summed up as follows:

1. That a dissociation of antibody from sensitized bacteria can be brought about by various methods and that an antibody extract or solution can be obtained free from any gross amount of serum protein.

2. That antibodies vary in their susceptibility to dissociation in the presence of sodium chlorid.

3. That the tonicity of the fluids employed during sensitization and dissociation appears to have some influence on the amount of antibody removed.

4. That, with the methods employed, a very considerable portion of the antibody remains attached to the bacteria.

From the practical view point it became necessary to attack the problem from two angles; to obtain a higher concentration of antibody in the final extracts and to devise a means of sterilization of the final product by filtration.

FACTORS INFLUENCING THE CONCENTRATION OF ANTIBODY IN THE FINAL EXTRACT

It is evident that the amount of antibody taken up by the antigen, the amount lost during washing and the efficiency of the final dissociation all have a bearing on the final concentration of antibody.

a. Factors influencing the degree of sensitization of antigens

1. Antigen. (a) Medium best suited for growth. (b) Use of live antigen. (c) Use of killed antigen; effect of heating the antigen on absorptive capacity. (d) Amount of antigen to use in relation to serum.

2. Sera. Although differences exist among immune sera as to their availability for this purpose, there seems to be no definite correlation between the values of the serum as measured by the protection test and their value for sensitization of antigen with subsequent dissociation. Accordingly, sera could only be selected by results.

3. Amount of sodium chlorid present during sensitization.

4. Tonicity of menstruum during sensitization.

5. Dilution of serum to be employed.
6. Time occupied in sensitization.
7. Temperature at which sensitization occurs.

b. Factors influencing the removal of antibody during the washing process

1. Tonicity of fluids employed.
2. Salt content of fluids employed.
3. Temperature of fluids, especially in regard to the temperature during the process of sensitization.

c. Factors influencing the final dissociation

1. Temperature. Influence on the amount of antibody dissociated; relation to temperature of sensitization.

2. Volume of menstruum employed.

3. Character of menstruum employed.

(a) Chemical factor; salt content. (b) Tonicity. (c) Reaction.

Since it was practically impossible to take up all these various points and establish the preferred method, experiments were undertaken to cover those that are considered essential.

Manipulation of antigen. Medium. The preferred medium for such work is one that will give a large amount of growth without the formation of a precipitate derived from the culture medium.

The reason for avoiding precipitate is obvious, since such a precipitate might be carried through the entire process and finally go into solution during the final dissociation.

Although in the case of pneumococcus, glucose serum media gave sufficiently heavy growths, these growths were always accompanied by a precipitate formation; hence a broth medium was devised and so handled as to preserve the growth factors (vitamines). This medium had a H ion content of pH 7.6 and from it the so called acid albumins had been previously precipitated. This proved a satisfactory medium, giving a

diffuse heavy growth with no traces of precipitate, and it was adopted as a standard during all the latter portion of our work.

Influence of heat on antigen. As has been shown, heat up to 100°C. can be applied to pneumococcus antigen without destroying its absorptive capacity and frequently antigens had been killed by heating at 55°C. to 60°C. more as a precaution against infection than in regard to the influence of these temperatures on the combining power.

In protocol 18, is shown the effect of various degrees of heat on pneumococcus antigen as influencing the amount of antibody dissociated.

Protocol 18. Influence of heating of antigen on extraction results

Antigens. Five day growth of virulent pneumococcus in special broth. The centrifuged sediment from this culture was emulsified in salt solution (one-third volume of original culture) and divided into three lots:

- A. Remained untreated.
- B. Heated at 65°C. for thirty minutes.
- C. Heated at 100°C. for ten minutes.

Serum. Polyvalent antipneumococcus.

Sensitization. A. Untreated antigen; 15 cc. added to 15 cc. of serum.

B. Antigen (65°C., thirty minutes); 15 cc. added to 15 cc. of serum.

C. Antigen (100°C., ten minutes); 15 cc. added to 15 cc. of serum.

All placed at 40°C. for two hours.

Washing. 1. The centrifuged sediments were each emulsified in 30 cc. of salt solution.

2. These emulsions and the centrifuged sediment were reemulsified in 30 cc. of distilled water.

3. The emulsions were then centrifuged and the supernatant fluids were discarded.

Dissociation. The washed sediments from A, B, and C were each emulsified in 15 cc. of salt solution. The emulsions were then placed at a temperature of 55°C. for thirty minutes. A portion of each emulsion was removed to be tested for potency. The remainder of each emulsion was centrifuged at high speed and the supernatant fluid was removed and tested for potency.

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULTS
	cc.		cc.	
Sensitized emulsions				
A. Antigen un- treated.....	0.2 0.2	Pneumococcus type I Pneumococcus type I	0.0001 0.001	S 70 70 70
B. 65°C. antigen...	0.2 0.2	Pneumococcus type I Pneumococcus type I	0.0001 0.001	S S S 18
C. 100°C. antigen..	0.2 0.2	Pneumococcus type I Pneumococcus type I	0.0001 0.001	S 70 36 70
Supernatant fluids				
A. (1) Antigen un- treated.....	0.2 0.2	Pneumococcus type I Pneumococcus type I	0.00001 0.0001	S 40 66 90
B. (1) 65°C. anti- gen.....	0.2 0.2 0.2	Pneumococcus type I Pneumococcus type I Pneumococcus type I	0.00001 0.0001 0.001	S S S S S 66
C. (1) 100°C. anti- gen.....	0.2 0.2 0.2	Pneumococcus type I Pneumococcus type I Pneumococcus type I	0.00001 0.0001 0.001	S S S 90 40 40
Original serum.....	0.2	Pneumococcus type I	0.01	S 90
Virulence control....	0	Pneumococcus type I	0.000000001	40 40

Summary and discussion. Two points are brought out in this experiment; first, that under the conditions employed, the use of antigen heated at 65°C. gives better final results than the use of unheated antigen or of antigen heated at higher temperatures. Two factors are involved. The amount of antibody combining with unheated antigen is probably greater than with heated antigen, but the union exhibits a greater resistance to the dissociation process. When boiled antigen is employed the amount of antibody combined is smaller but dissociation takes place more readily. With the antigen heated at 65°C. a balance is found where the amount of antibody combined is relatively large and dissociation takes place readily. The second point is that the emulsions of the treated bacteria give at least as high a protection as the centrifuged supernatant fluids. Previous experiment had shown more marked differences in this respect, which means that antibody may be combined with antigen so as to remain attached in the test-tube and yet be readily given up in the animal

body. A large number of experiments in this connection have shown that passive immunity is conferred by sensitized bacteria.

Influence of salt concentration (or tonicity) on sensitization

Since agglutinins, as well as protective antibody, were present in our sera and many of our emulsions agglutinated promptly on the addition of serum, means were sought to hinder this action since it reduced the surface of the antigen exposed to the action of the serum. Advantage was taken of the well known fact that absence of salt hinders the flocculation in the agglutination reaction.

The emulsions of centrifuged organisms were prepared in distilled water in place of salt solution; and the serum was added in such proportions as to avoid precipitation of globulin. The subsequent washing of the antigen was carried out with dilutions of salt of the same tonicity as was present in the sensitization fluid.

An experiment bearing on this point is shown in protocol 19.

Protocol 19. Influence of the reduction of the salt content during sensitization on the amount of final extraction of antibody

Serum. Antipneumococcic.

Antigen. One week's growth of pneumococcus type I in special broth. Two 40 cc. lots placed in centrifuge tubes and centrifuged at high speed. To the centrifuged sediments was added:

A. 15 cc. of salt solution.

B. 15 cc. of distilled water.

Sensitization. To each (A and B) emulsion were added 5 cc. of serum and these mixtures were placed at 40°C. for two hours; they were then centrifuged and the supernatant fluids were discarded.

Washing. A. Centrifuged sediment washed once with 20 cc. of salt solution and once with 20 cc. of distilled water.

B. Centrifuged sediment washed twice with 20 cc. of one-fourth strength physiological salt solution. (Salt content equal to the amount present during the sensitization of B.)

Dissociation. The washed sediments (A and B) were emulsified in 10 cc. of salt solution and placed at 55°C. for thirty minutes. Both mixtures were then centrifuged and the final supernatant fluids were reserved for testing.

Protection tests

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULTS
	cc.		cc.	
Original serum	0.2	Pneumococcus type I	0.05	S 90
	0.2	Pneumococcus type I	0.1	18 70
A. Extract (sensitized in physiological salt solution, 3 parts; serum, 1 part)	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	40 40
	0.2	Pneumococcus type I	0.01	18 18
B. Extract (sensitized in distilled water, 3 parts; serum, 1 part) {	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	S S
	0.2	Pneumococcus type I	0.01	40 70
Virulence control		Pneumococcus type I	0.000000001	40 40

The results of this experiment show a difference in favor of employing distilled water for emulsifying the antigen to be employed in the original sensitization. These results were confirmed and this technic was employed for several experiments, but it was finally abandoned in favor of salt solution emulsions for two reasons; first, because no assurance could be had that a certain amount of globulin was not precipitated from the serum, and secondly, because it was felt that often the distilled water caused an extraction of the pneumococci with the freeing of a precipitinogen, which reacted with the precipitin known to be present in the serum. Both of these reactions were to be avoided if we wished to obtain extracts reasonably free from serum proteins.

Time of exposure during sensitization. No direct comparative experiments were done on this phase of the problem, but periods from one to twenty-four hours were used, depending on the temperature employed.

Temperature of sensitization. This factor seems to be more important in its relation to the other steps in the dissociation technic than in the question of obtaining an adequate combination with antibody.

Landsteiner and Jagic (13) believed, on the basis of very few experiments, that the temperature at which sensitization took

place was a significant factor. They demonstrated with hemagglutinins that, when measured by the absorption test, less combination took place at 47°C. than at room temperature, and from this fact they deduced that the difference in temperature at which the sensitization took place and that employed for dissociation was the important factor. Three points are to be considered in this connection:

First, do as many antibodies attach themselves to the antigen at low (5°C. to 10°C) as at higher temperatures; secondly, if so, is an unusually large proportion lost during the washing process if the temperature of the wash fluids is higher than that at which sensitization took place; and thirdly, is the amount of antibody dissociated proportional to the range of temperature between sensitization and dissociation?

We had been accustomed to employ for sensitization, temperatures from 37° to 50°C. but in many instances we had, as a matter of convenience, placed such mixtures in the ice box at 5°C. for periods of from 18 hours to several days.

A review of forty of our experiments in regard to the above points shows:

1. That sensitization (combination of antigen and antibody) is as effective at low temperatures as at high, if continued for a considerable period.

2. That washing fluids, either salt solution or distilled water, used at a temperature above that of the sensitization contain more antibody than can be accounted for by the progressive dilution of any serum remaining attached to the antigen.

3. That an extreme range of temperature between the sensitizing period and the dissociation period is not necessary for efficient extraction.

Relation of amount of antigen to the serum employed. As a result of numerous experiments, a relation of approximately ten to twelve billion organisms to the cubic centimeter of serum was adopted as giving the best results.

Temperature of washing fluids. As indicated above, wash fluids at a higher temperature than that at which sensitization took place, tend to remove a considerable portion of the attached

antibody. It also seemed wise to employ the same tonicity in such fluids as was present during sensitization.

Temperature of dissociation fluids and relation to temperature of sensitization. The temperature at which dissociation occurs has a bearing both on the amount of antibody removed and on the subsequent viability of this antibody.

Following the work of Kosakai (19) and our own determinations with agglutinin (protocol 5) we had employed, as routine, a temperature of 55°C., for thirty minutes.

However, Landsteiner and Jagic (13) had shown that with normal hemagglutinins a temperature of 42°C. to 45°C. was superior to higher temperatures. Since we considered that the antibody might be somewhat injured by the higher temperatures, comparative observations were made on this point.

Protocol 20. (1) Dissociation at temperatures of 42°C. and 55°C. (2) Variation of amounts of sodium chlorid during sensitization

Serum. Antipneumococcic.

Antigen. Four sets of 40 cc. each of pneumococcus type 1 culture.

The antigen sets were centrifuged and the sediments emulsified as follows:

1. In 10 cc. distilled water.
2. In 10 cc. distilled water.
3. In 10 cc. salt solution.
4. In 10 cc. salt solution.

Sensitization. To each emulsion were added 5 cc. of serum and the mixtures were placed at 40°C. for thirty minutes. Each of these was then centrifuged and the supernatant fluid was discarded.

Washing. The centrifuged sediments were washed as follows:

- 1 and 3. Twice with one-fourth strength physiological salt solution.
- 2 and 4. Twice with physiological salt solution.

Dissociation. The washed sediments were each emulsified in 5 cc. of physiological salt solution and heated as follows:

- 1 and 4. To 42°C. for thirty minutes.
- 2 and 3. To 55°C. for thirty minutes.

All sets were then centrifuged and the clear supernatant fluids were tested for the presence of protective antibodies.

Protection tests

EXTRACTS	AMOUNT	CULTURE	AMOUNT	RESULTS
	cc.		cc.	
Extract 1, 42°C. set.	0.2	Pneumococcus type I	0.001	S 64
	0.2	Pneumococcus type I	0.01	S 17
Extract 2, 55°C. set.	0.2	Pneumococcus type I	0.001	S S
	0.2	Pneumococcus type I	0.01	64 17
Extract 3, 42°C. set.	0.2	Pneumococcus type I	0.001	S 48
	0.2	Pneumococcus type I	0.01	18 17
Extract 4, 55°C. set.	0.2	Pneumococcus type I	0.001	S 40
	0.2	Pneumococcus type I	0.01	S 17

The results with this experiment, while somewhat irregular, show that, under the conditions employed, very little difference in the amount of antibody existed between the sets extracted at 42°C. and those at 55°C. If consistent results could be obtained with 42°C., it was felt that such a temperature would in reality injure the antibody less than 55°C.

The results obtained also showed little difference as to the effect produced by variations in the salt content between the sensitizing fluid and that employed for dissociation, contrary to the results seen in protocol 17.

An experiment done in a similar manner, but with the use of a pneumococcus type 2 serum and antigen showed little differences in effect between 42°C. and 55°C. in respect to the amount of antibody dissociated.

Volume of dissociation fluids in relation to the volume of the sensitizing serum. In the preliminary work with agglutinins, our results tended to confirm those of Kosakai (19), with hemolytic amboceptor, that the amount of antibody dissociated was in proportion to an increase in volume of the fluid used for this purpose and we regarded this as an evidence of the influence of the dilution of electrolyte.

In subsequent tests, however, this factor did not seem to bear the same relationship, particularly when mild alkalis were present.

Influence of the character of the fluid employed for dissociation: Sodium chlorid content. Although our own experiments with agglu-

tinins and those of Kosakai (19) with hemolytic amboceptor have apparently demonstrated that dissociation was proportioned to the reduction of the sodium chlorid content, our experiments with bactericidal antibody and the protective antibody from pneumococcus sera show that with these antibodies, the presence or absence of sodium chloride, does not influence the dissociation in any marked degree.

Tonicity. It has been shown that dissociation occurs in distilled water, in physiological salt solution and in isotonic solutions of sugar; another experiment, bearing on this point, was done in which a comparison was made between dissociation in salt solution 0.85 per cent and in salt solution 6.8 per cent which showed little difference in the amounts of antibody recovered. It must, therefore, be considered that tonicity has little or no influence on dissociation.

Reaction. Our experiment shown in protocol 6, Gay's (9) observation on the effect of alkali on antibody and our desire to obtain solutions proper for intravenous use, had led us to neglect this phase of the question.

In the course of experiments in which a solution of ammonium carbonate was employed as a dissociation agent, we made the discovery that the antibody solutions containing this agent were filterable and this at once suggested that other mildly alkaline substances might have the same influence.

An investigation of the influence of solutions of sodium bicarbonate proved this to be the case.

The amounts of antibody recovered are not more than can be recovered by several other methods but, contrary to the experience with other solutions, the antibody is freely filterable.

A discussion of the phenomenon of dissociation will be taken up under the general summary.

Concentration of antibody extracts. At the suggestion of D. Van Slyke ammonium carbonate solutions were employed as dissociation agents since this substance in the presence of a vacuum would in itself dissociate, enabling us to secure concentrated solutions of antibody without at the same time concentrating the salt content.

An experiment utilizing ammonium carbonate is shown in protocol 21.

Protocol 21. (1) Comparison of dissociation in salt solution and ammonium carbonate solution. (2) Influence of ammonium carbonate on the filterability of antibody

Equal portions of sensitized and washed pneumococcus antigen were emulsified in:

A. 800 cc. of salt solution.

B. 800 cc. of a 2 per cent solution of ammonium carbonate in distilled water.

Strength of emulsions, 5 billion organisms to 1.0 cc.

Dissociation. The A emulsion was heated to 55°C. for twenty minutes, placed on ice over night, reheated to 55°C. for fifteen minutes and centrifuged; the supernatant fluid was removed and designated as "salt extract centrifuged."

The B emulsion was heated to 40°C. and exposed to the action of a water vacuum pump until all visible dissociation of the ammonium carbonate ceased; it was then centrifuged and the supernatant fluid was tested as follows:

1. Tested for ammonium carbonate content. One per cent was found still present.

2. 20 cc. were removed for protection test under the designation "ammonium carbonate centrifuged."

3. The remainder was filtered through a filter candle and designated as "Ammonium carbonate filtered."

Protection tests

EXTRACTS	AMOUNT	CULTURE	AMOUNT	RESULTS
	cc.		cc.	
Salt extract centri- fuged.....	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	S 48
	0.2	Pneumococcus type I	0.01	X 43
Ammonium carbonate centrifuged.....	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	S 64
	0.2	Pneumococcus type I	0.01	X 20
Ammonium carbonate extract filtered.....	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	S 38
	0.2	Pneumococcus type I	0.01	X 20
Virulence control.....		Pneumococcus type I	0.000000001	77 41

Summary. 1. Dissociation in salt solution and ammonium carbonate solution proved equal in amount.

2. In the presence of 1 per cent ammonium carbonate, antibody is filterable.

Influence of reduced ammonium carbonate content on filterability. 700 cc. of the filtered product described above was attached to a powerful oil vacuum pump at a temperature of 37°C. to 42°C. at intervals over a period of seventy-two hours.

The dissociation of the carbonate was continued until tests showed a percentage present of less than 0.125 per cent.

This was accompanied by the formation of a flocculent precipitate and a concentration of volume to 250 cc.

This product was centrifuged.

The supernatant fluid was refiltered through a filter candle and the sediment was shaken up in 20 cc. of salt solution; both solutions were tested for antibody content.

Protection tests

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULTS
	cc.		cc.	
Refiltered supernatant fluid....	0.2	Pneumococcus type I	0.00001	64
	0.2	Pneumococcus type I	0.0001	52
	0.2	Pneumococcus type I	0.001	52
Sediment emulsified in salt solution.....	0.2	Pneumococcus type I	0.0001	S
	0.2	Pneumococcus type I	0.001	S
	0.2	Pneumococcus type I	0.01	36

It is evident that, with a reduction of the ammonium carbonate content below a certain point, some of the antibody is precipitated or is removed by the precipitate which forms, and that the antibodies remaining in the supernatant fluid are no longer filterable.

The results obtained in protocol 21, are evidence that when the ammonium carbonate content is reduced below a certain point, the antibody is no longer filterable, but that when as much as 1 per cent is still present, it remains free and can pass the filter.

On injection of 5 cc. of this filtered solution into guinea-pigs several died of ammonium carbonate poisoning. Hence, if this material was to be employed, it became necessary to find a con-

centration which would allow of filtration but which would not be poisonous.

A second experiment on the influence of the reduction of the ammonium carbonate content on filterability (see protocol 23) confirmed the results in protocol 21.

In protocol 22 is seen the results of evaporating the extraction fluids to dryness and taking up the residue in salt solution and distilled water.

Protocol 22. (1) *Influence of drying on antibody.* (2) *Methods of concentration.* (3) *Nature of antibody*

Antigen. Distilled water emulsion of pneumococci type I (10 billion to 1.0 cc.).

Serum. Antipneumococcic polyvalent.

Sensitization. 200 cc. of serum were added to 200 cc. of antigen. Temperature of sensitization 5°C. Time of sensitization five hours. The mixture was centrifuged and the supernatant fluid was discarded. The centrifuged sediment was emulsified in 200 cc. of ice cold salt solution (0.4 per cent), and recentrifuged and the sediment was again emulsified in 200 cc. of cold salt solution; the washing was repeated a third time.

Dissociation. The washed sediment was emulsified in 250 cc. of a 2 per cent solution of ammonium carbonate in distilled water (at a temperature of 42°C.), heated to 42°C. for fifteen minutes, then attached to a vacuum pump for two hours during which a vigorous dissociation of the ammonium carbonate took place.

The emulsion was centrifuged and the supernatant fluid was divided into two portions: A, designated as "centrifuged extract;" B, filtered through a filter candle, and then designated as "filtered extract."

A test of the ammonium carbonate content of the filtered extract showed more than 1 per cent still present; accordingly, dissociation of ammonium carbonate was continued. Drying and concentration of antibody was affected by attaching 15 cc. of the "filtered extract" to the vacuum pump and keeping it at a temperature of 40°C. (evaporated to dryness in two hours).

The dried material was taken up in 5 cc. of distilled water (one-third original volume). It was then centrifuged and the supernatant fluid was designated as "concentrated antibody C." 225 cc. of the same "filtered extract" was evaporated to dryness during a period of two days. The residue was taken up in 20 cc. of salt solution and designated as "concentrated antibody D."

Protection test

(The following table is made up of tests on three days since the virulence of the strain employed was the same on each day.)

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULTS
	cc.		cc.	
A. Centrifuged extract.....	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	65 51
B. Filtered extract...	0.2	Pneumococcus type I	0.0001	S 51
	0.2	Pneumococcus type I	0.001	S 51
	0.2	Pneumococcus type I	0.01	51 51
C. Concentrated antibody (3x).....	0.2	Pneumococcus type I	0.0001	S
	0.2	Pneumococcus type I	0.001	S
	0.2	Pneumococcus type I	0.01	30
D. Concentrated antibody (10x).....	0.2	Pneumococcus type I	0.0001	S
	0.2	Pneumococcus type I	0.001	S
	0.2	Pneumococcus type I	0.01	S
	0.2	Pneumococcus type I	0.1	10
Virulence control.....		Pneumococcus type I	0.000000001	70 82

In this experiment it is shown that little deterioration of the antibody occurs during drying under the conditions employed and, if we can ignore the possibility of small amounts of ammonium carbonate remaining undissociated, it may be inferred that the antibody is not of a euglobulin nature since it is taken up by distilled water. In the following protocol 23, a comparison of the dissociation in a solution of ammonium carbonate and in sodium bicarbonate is made.

Protocol 23. Dissociation by means of ammonium carbonate and sodium bicarbonate solutions. Filterability of such extracts

1. *Antigen.* Distilled water emulsion of pneumococcus type I (strength eight billion per cubic centimeter).

2. *Serum.* Antipneumococcic.

Sensitization. 600 cc. of antigen + 600 cc. of serum were placed at 5°C. for seventy-two hours. The mixtures were centrifuged.

Washing. The centrifuged sediment was emulsified in 900 cc. of salt solution and recentrifuged; the sediment was again emulsified in 300 cc. of salt solution and divided into two equal portions (A and B). The two

lots were recentrifuged and the sediments were treated as described below.

Dissociation. The washed sediment of A was emulsified in 300 cc. of a 0.2 per cent solution of ammonium carbonate in distilled water. The mixture was heated to 30°C. and attached to a vacuum pump; this action was continued until the ammonium carbonate content was reduced below 0.125 per cent.

The material was centrifuged and the supernatant fluid was divided into two portions. One lot, amounting to 20 cc., was designated as "centrifuged extract A." The remainder was filtered through a filter candle and designated as "filtered extract A."

The washed sediment of B was emulsified in a 0.5 per cent solution of sodium bicarbonate in 0.85 per cent salt solution and placed at room temperature for one hour and at 30°C. for one hour. The mixture was centrifuged and the supernatant fluid was divided into two portions. One was designated as "centrifuged extract B;" the second, after passage through a filter candle, was designated as "filtered extract B."

These extracts were tested as to their protective antibody content.

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULTS
	cc.		cc.	
Ammonium carbonate set				
Centrifuged extract A. . .	0.2	Pneumococcus type I	0.0001	S
	0.2	Pneumococcus type I	0.001	S
	0.2	Pneumococcus type I	0.01	18
Filtered extract A.	0.2	Pneumococcus type I	0.0001	57
	0.2	Pneumococcus type I	0.001	32
	0.2	Pneumococcus type I	0.01	12
Sodium bicarbonate set				
Centrifuged extract B. . .	0.2	Pneumococcus type I	0.0001	S
	0.2	Pneumococcus type I	0.001	S
	0.2	Pneumococcus type I	0.01	32
Filtered extract B.	0.2	Pneumococcus type I	0.0001	S
	0.2	Pneumococcus type I	0.001	S
	0.2	Pneumococcus type I	0.01	32
Control serum.	0.2	Pneumococcus type I	0.01	S
	0.2	Pneumococcus type I	0.1	34
Virulence controls.		Pneumococcus type I	0.00000001	82
		Pneumococcus type I	0.00000001	34

The results obtained in protocol 23 are sufficiently clear cut to show:

1. That a reduction of the ammonium carbonate content below 0.125 per cent with a consequent reduction in the alkalinity of the product causes the antibody to become unfilterable.

2. That a solution of 0.5 per cent of sodium bicarbonate in salt solution is not only as good a dissociation agent, but renders the product filterable. This latter result was repeatedly confirmed, and it was found that such solutions could be repeatedly filtered with no loss of antibody content.

From a practical viewpoint this observation was of the greatest value since it enabled us to produce a sterile end product, and the presence of a small percentage of sodium bicarbonate was not contra-indicated in solutions intended for intravenous use, but was to be desired.

From the theoretical viewpoint also such a phenomenon is of great interest since it indicates that a change in the condition of the antibody has taken place.

It had been previously considered by us that, in the salt solution extracts of sensitized antigen, the antibody present was probably in loose combination with the bacterial extracted substance, held in suspension or colloidal solution, and that in filtration the removal of a large part of this bacterial matter carried with it the attached antibody. That in the sodium bicarbonate extractions a certain amount of material removed from the bacteria employed does go into solution is evident from the appearance of the filtered products.

If such products are filtered at a temperature of 30°C. or over, a perfectly clear solution is obtained, but after standing on ice for twenty-four hours such solutions become opalescent, or may even precipitate slightly. On reheating the opalescence disappears.

If the ice cold solution is filtered, the opalescence is removed and the solutions remain clear. Such filtration is not followed by loss of antibody; hence, it can be stated that the antibody was not in combination with the material and that it is probable that in the presence of this small amount of sodium bicarbonate

the antibodies content detectable by the protection test are in a free condition.

However, the possibility of a reversal of the electrical charge of the antibody must be borne in mind. In a neutral solution such as salt solution the antibody has a charge which is opposite to that of the filter and this may account for the antibody removal.

GENERAL SUMMARY AND CONCLUSIONS

It has been shown in the preceding pages that antibody which has once combined with its homologous antigen can be removed from its attachment and rendered reavailable.

That such a reversal of the reaction is never complete. This has been demonstrated for agglutinins, bactericidal antibodies, and the protective antibodies in antipneumococcic serum. It has also been shown by us that agglutinins and by Kosakai (19) that hemolytic amboceptor differs from the bactericidal antibody and the protective antibody in that, in the presence of sodium chloride, dissociation does not occur in the same degree as in solutions not containing this salt.

It has been shown further that the protective antibody attached to antigen can be rendered again available by treatment with 10 per cent saccharose solutions, 5.6 per cent dextrose solutions, distilled water, normal salt solutions, ammonium carbonate solutions and salt solution containing a small amount of sodium bicarbonate.

We have also suggested the possibility that the antibody, although available in the presence of fresh antigen (as shown by the protection test), is probably not, in the strict sense of the word, free, but attached to the remains of bacterial structures still present in the solutions; this holds for all solutions, with the exception of those containing a small amount of alkali such as ammonium carbonate and sodium bicarbonate.

The latter are freely filterable, passing through filter candles without a loss of antibody content, whereas all other solutions tried showed a considerable loss of antibody on such filtration.

The attempt to prove the mechanism of the dissociation phenomenon shows that it cannot be attributed alone to the

presence or the absence of sodium chlorid, to changes in the tonic conditions surrounding the sensitized organisms, or to wide changes in temperature conditions, although each of these factors seems to have some bearing, with temperature changes having the most influence.

The reasons underlying the mode of dissociation of such complexes, as we have been dealing with, are difficult to determine. Why, if antibody is really freed during the process used, does it not immediately recombine with the antigen present? We have presented evidence that this recombination does occur, but slowly, and this leaves three suppositions; that the combining power of the antibody has been diminished, or that a third factor is responsible.

Although, up to this time, we had failed to demonstrate the mechanism underlying the dissociation phenomenon, a review of all the facts at our disposal led us to the conclusion that there was another factor, probably associated in the serum, the presence of which acted as a binder and the absence of which, when removed by washing (progressive dilution), allowed dissociation to take place.

Such a factor could be specific or non-specific, organic or inorganic. A series of experiments to determine this point were undertaken.

Protocol 24. Influence on the dissociation of protective antibody of normal serum and heterologous immune serum, in comparison with physiological salt solution

Three lots of antigen, each consisting of 80 cc. of a twenty-four hour growth in broth of pneumococcus type 1, were centrifuged at high speed. The sediments were each washed once with 40 cc. of salt solution and finally each was taken up in 10 cc. of salt solution.

Serum. Antipneumococcic 81667—polyvalent horse serum.

Sensitization. To each 10 cc. lot of antigen were added 5 cc. of the immune pneumococcus serum. The temperature of sensitization was 40° to 45°C., and the time, one hour. The mixtures were then centrifuged and the supernatant fluids were discarded.

Washing. The sensitized bacteria were emulsified and washed with 40 cc. of salt solution.

Dissociation. The washed sensitized bacteria were treated as follows:

Lot A was emulsified in 10 cc. of physiological salt solution.

Lot B was emulsified in 10 cc. of a mixture of one part normal horse serum and two parts of salt solution. (The dilution of the serum was the same as that at which sensitization had occurred.)

Lot C was emulsified in 10 cc. of a mixture of one part of immune antimeningococcic horse serum and two parts of salt solution.

Lots A, B, C, were then placed in a water bath at a temperature of 50° to 55°C. for one hour.

All were centrifuged at high speed, and the clear supernatant fluids were removed and tested for protective antibody content.

DISSOCIATION FLUID	AMOUNT	PNEUMOCOCCUS TYPE I	TEST	RETEST
	cc.	cc.		
A. Salt solution lot.	0.2	0.00001	S	S
	0.2	0.0001	48	S
	0.2	0.001	S	S
	0.2	0.01	24	18
B. Normal serum lot.	0.2	0.00001	24	S
	0.2	0.0001	40	20
	0.2	0.001	40	18
	0.2	0.01	18	18
C. Heterologous immune serum lot.	0.2	0.00001	S	72
	0.2	0.0001	40	20
	0.2	0.001	25	18
	0.2	0.01	18	18
Virulence control.		0.000000001	S S	24 19
		0.00000001	44 44	40 25
		0.0000001	28 25	25 24

The results of the above experiment show that dissociation is much diminished in the presence of both normal serum and heterologous immune serum, and they indicate that the preventive factor is non-specific.

If such a factor is organic, it renders the determination of its nature practically impossible, but if one of the inorganic salts present in blood serum is responsible, such determinations can be made.

The following protocol 25, shows the differences in the influence of soluble and comparatively insoluble calcium salts on the dissociation of the protective antibody from sensitized antigen.

Protocol 25. Comparison of the influence of sodium chlorid, sodium chlorid plus calcium sulphate and sodium chlorid plus calcium chlorid on the dissociation of the protective antibody.

Preparation of the fluids employed for dissociation. To 500 cc. of physiological salt solution were added 0.131 gram of calcium sulphate. (Twice the equivalent amount of calcium present in blood serum.) To 500 cc. of physiological salt solution was added 0.106 gram of calcium chloride (twice the equivalent amount of calcium present in blood serum.) Schmidt's analysis.

Antigen. The type I pneumococci, centrifuged from 240 cc. of a forty-eight hour growth in "hormone" broth, were taken up in 36 cc. of salt solution and divided into three portions of 12 cc. each.

Serum. 81667; polyvalent antipneumococcic horse serum.

Sensitization. To each portion of 12 cc. of antigen were added 10 cc. of serum. The temperature of sensitization was 52°C. and the time of sensitization one hour. The three lots were centrifuged and the supernatant fluids were discarded.

Washing. Each centrifuged sediment was emulsified in 30 cc. of salt solution and recentrifuged.

Dissociation. The washed sediments were treated as follows:

A. Emulsified in 10 cc. of physiological salt solution.

B. Emulsified in 10 cc. of salt solution containing calcium sulphate.

C. Emulsified in 10 cc. of salt solution containing calcium chlorid.

A, B, and C were placed in a water bath at 53°C. for 1 hour with frequent shakings.

A, B, and C were centrifuged, the supernatant fluids were removed and placed on test to determine the protective antibody content.

The following experiment was carried out to control the direct effect of the calcium sulphate on the protective test.

Protection tests on mice

PRODUCT	AMOUNT	CULTURE	AMOUNT	RESULTS
	cc.		cc.	
A. Salt solution dissociation.....	0.2	Pneumococcus type I	0.00001	S S
	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	S S
	0.2	Pneumococcus type I	0.01	S 64
B. Salt solution plus calcium sulphate dissociation.....	0.2	Pneumococcus type I	0.00001	S S
	0.2	Pneumococcus type I	0.0001	39 62
	0.2	Pneumococcus type I	0.001	51 47
	0.2	Pneumococcus type I	0.01	39 47
C. Salt solution plus calcium chlorid dissociation.....	0.2	Pneumococcus type I	0.00001	S
	0.2	Pneumococcus type I	0.0001	S
	0.2	Pneumococcus type I	0.001	S
	0.2	Pneumococcus type I	0.01	S
Virulence control.....		Pneumococcus type I	0.000000001	39 39
		Pneumococcus type I	0.00000001	39 39

Equal portions of the A (salt solution) lot and the original solution of calcium sulphate were mixed and placed on test in a similar manner, 0.4 cc. being used in place of 0.2 cc. for the injection.

The mice thus received comparable dosage of antibody and calcium sulphate.

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULTS
	cc.		cc.	
Mixture of A salt solution dissociation lot and calcium sulphate solution.....	0.4	Pneumococcus type I	0.00001	S S
	0.4	Pneumococcus type I	0.0001	S S
	0.4	Pneumococcus type I	0.001	S 47
	0.4	Pneumococcus type I	0.01	S 62
Virulence control.....		Pneumococcus type I	0.000000001	S S
		Pneumococcus type I	0.00000001	47 47

The results in protocol 25, indicate that the presence of calcium sulphate prevents in some manner an adequate dissociation, since the control set shows little direct effect of this salt on the death of injected mice and that further this restraining effect is not apparent in the calcium chloride set.

A further experiment done in a similar manner to determine whether the failure of dissociation was due to the presence of sulphates as such (such as potassium and magnesium sulphate) or was confined to calcium sulphate, shows that as good a dissociation occurs in isotonic solutions of potassium sulphate, magnesium sulphate, or calcium chlorid as occurred in physiological salt solution.

Since calcium exists in blood serum mostly as calcium phosphate, experiments were designed to determine the influence of this salt on the dissociation phenomenon.

In protocol 26 is seen the influence of calcium phosphate on dissociation.

Protocol 26. Influence of calcium phosphate on the dissociation of pneumococcus protective antibody

Preparation of calcium phosphate solution. To 1000 cc. of salt solution was added 0.6 gram of calcium phosphate, $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$. (Twice the amount present in 1000 cc. of normal serum according to Schmidt's analysis.) This went only partially into solution and was stood at room temperature for forty-eight hours to settle. A portion of the clear supernatant fluid was removed and employed for dissociation purposes.

Note. A test of the supernatant by the addition of potassium oxalate showed the presence of calcium salt in solution.

Sensitization. Antigen. Two portions 12.5 cc. each (10,000 million per cc.) pneumococcus type 1. To each portion was added 12.5 cc. of antipneumococcic horse serum. The temperature of sensitization was 55°C . and the time of sensitization, 1 hour. The mixtures were frequently shaken. Both portions were then centrifuged.

Washing. The centrifuged sediments were each emulsified in and washed twice with 40 cc. of ice cold salt solution.

Dissociation. The washed sediments were severally treated as follows:

1. Emulsified in 12.5 cc. of salt solution.
2. Emulsified in 12.5 cc. of salt solution containing calcium phosphate.

Both were placed in a water bath at a temperature of 55°C . for thirty minutes, and then centrifuged and the supernatant fluids removed for testing.

Protection tests

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	MICE	
	cc.		cc.		
"1" salt solution dissociation.....	0.2	Pneumococcus type I	0.0001	S	S
	0.2	Pneumococcus type I	0.001	S	S
	0.2	Pneumococcus type I	0.01	42	42
"2" salt solution and calcium phosphate dissociation.....	0.2	Pneumococcus type I	0.00001	44	24
	0.2	Pneumococcus type I	0.0001	96	18
	0.2	Pneumococcus type I	0.001	42	22
	0.2	Pneumococcus type I	0.01	25	18
"3" control*.....	0.4	Pneumococcus type I	0.0001	S	S
	0.4	Pneumococcus type I	0.001	66	42
	0.4	Pneumococcus type I	0.01	22	21
Virulence control.....		Pneumococcus type I	0.00000001	S	S
		Pneumococcus type I	0.0000001	42	42

* To control the direct effect of calcium phosphate, equal portions of the "1" salt solution extraction and salt solution containing calcium phosphate were mixed. Since double the usual dose was given, the mice received the same number of antibodies as in "1" and the same amount of calcium phosphate as in "2."

It will be noted that the presence of calcium phosphate apparently reduced the value of the extract "1."

Repeated tests with normal serum had shown that dissociation was always diminished when a considerable quantity of serum was present. The tests with calcium sulphate and calcium phosphate had indicated that the comparatively insoluble calcium salts might be the responsible factors, and the experiment shown in protocol 27 was designed to determine this point.

Protocol 27. Influence of the removal of the calcium salts from normal horse serum on the dissociation value of such serum

Removal of calcium salts.

Serum. Normal horse serum.

To 25 cc. of clear serum was added 0.1 cc. of a 10 per cent solution of potassium oxalate. This was allowed to stand two hours at room temperature and then centrifuged at high speed.

The clear supernatant fluid was removed and employed for dissociation purposes.

The following test was made to furnish proof of the removal of calcium salts. 1 cc. of clear supernatant fluid was added to 1 cc. of 10 per cent

potassium oxalate solution. No precipitate in eighteen hours indicated absence of calcium. 1 cc. of clear supernatant fluid was added to 1 cc. of an isotonic solution of calcium chlorid. An immediate precipitate indicated the presence of an excess of potassium oxalate.

Sensitization of antigen. To each of three (15 cc.) portions of pneumococcus type 1 antigen (20,000 million per cubic centimeter) was added 15 cc. of antipneumococcic horse serum. The dilution of the serum during sensitization was 1:2; the temperature of sensitization 48°C.; the time of sensitization, one hour.

Resensitization. The centrifuged sediments from the above sets were reemulsified, each in 15 cc. of salt solution, and 15 cc. of the same antipneumococcic serum added.

The dilution of the serum during resensitization was 1:2; the temperature, 48° to 10°C.; the time, two hours to sixteen hours.

Washing. The centrifuged sediments were each emulsified in and washed twice with 40 cc. of ice cold salt solution.

Dissociation. The washed sediments were severally treated as follows:

1. Emulsified in 15 cc. of physiological salt solution.
2. Emulsified in 15 cc. of a mixture of equal parts of the normal serum and salt solution. (Dilution of serum (1:2) the same as during sensitization.)
3. Emulsified in 15 cc. of a mixture of equal parts of the oxalated normal serum (free from calcium) and salt solution. (Dilution of serum (1:2), the same as during sensitization.)

The temperature at which dissociation occurred was 55°C.; the time of dissociation was thirty minutes. The three lots were centrifuged and the clear supernatant fluids were removed.

Preparation of controls. Since it was necessary to control the influence of serum, as such, on the absorption of antibody from the peritoneum and also to control the poisonous effect of the potassium oxalate remaining in solution, the following controls were introduced.

4. A mixture of equal parts of "1" salt solution extract and of untreated normal serum.
5. A mixture of equal parts of "1" salt solution extract and of oxalated serum.

The use of twice the usual amounts of these mixtures in the protective tests gives in "4" the same number of antibodies as in "1" and the same amount of serum as in "2."

In "5" the same number of antibodies were present as in "1" and the same amount of serum and potassium oxalate as in "3."

Protection tests

TEST MATERIAL	AMOUNT	CULTURE	AMOUNT	RESULTS
	cc.		cc.	
Dissociated extracts				
1. Salt solution extract...	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	S 40
	0.2	Pneumococcus type I	0.01	S 92
2. Untreated normal serum extract.....	0.2	Pneumococcus type I	0.00001	S S
	0.2	Pneumococcus type I	0.0001	S 45
	0.2	Pneumococcus type I	0.001	74 40
	0.2	Pneumococcus type I	0.01	43 21
3. Oxalated serum extract.	0.2	Pneumococcus type I	0.0001	S S
	0.2	Pneumococcus type I	0.001	S 46
	0.2	Pneumococcus type I	0.01	42 26
Controls				
4. Salt solution extract and untreated normal serum.	0.4	Pneumococcus type I	0.0001	S S
	0.4	Pneumococcus type I	0.001	S 40
	0.4	Pneumococcus type I	0.01	S 74
5. Salt solution extract and oxalated normal serum..	0.4	Pneumococcus type I	0.0001	S S
	0.4	Pneumococcus type I	0.001	S 74
	0.4	Pneumococcus type I	0.01	74 43

The results in the above experiment, while not as striking as those in protocol 26, gives some fairly definite evidence that the salts precipitated from serum by potassium oxalate were the factors concerned in preventing an adequate dissociation of the combination of pneumococcus antigen and the pneumococcus protective antibody in a menstruum of normal serum.

Kosakai (19) has shown that sodium chlorid exercises this inhibiting influence on the dissociation of hemolytic amboceptor and we have previously shown the similar influence of sodium chloride on the dissociation of agglutinins and the failure of sodium chloride to influence in the same degree the dissociation of the bactericidal antibody-antigen combination.

Those who have worked with the dissociation of the antigen-

antibody complex have been concerned in general with proving that such dissociation took place.

Landsteiner and Jagic (13) advanced a purely chemical theory to account for this phenomenon. Kosakai (19) attributed it to the dilution of electrolyte as such.

Neither theory can be substantiated, that of Landsteiner for lack of evidence and that of Kosakai by reason of the fact that the presence of electrolyte as such does not hinder the dissociation of certain antibodies.

Although the evidence accumulated is as yet too slight to allow of the formulation of general laws, yet it is probable that further work will establish the relationships of the various antibodies to different salts. (As shown above for the protective antibody.)

When such relationships are determined a consideration of all the facts, governing both the combinations and dissociation of antigen and antibody, will enable the setting forth of an adequate conception of such phenomena.

At present, the most that can be safely said in connection with the phenomenon of dissociation of the antibody-antigen combination is that *apparently* it is governed by the dilution of the *binding salt* present, and that the nature of this *binding salt* differs for different antibodies.

In any case, the conception of such *binding salts* offers a different angle from which to attack the problem of the nature of the antigen-antibody combination.

A theory based on the assumption that dilution of the *binding salt* is responsible for a reversal of the antigen-antibody combination and that such *binding salts* differ for different antibodies will explain many of the facts noted in such dissociation work. For example:

1. The comparative failure of the dissociation of agglutinin and hemolytic amboceptor in salt solution and their dissociation in solutions not containing this salt.
2. The dissociation of protective antibody and bactericidal antibody in salt solution.
3. The influence of heat in hastening the reaction.

4. The influence of time when dissociation fluids are kept at low temperatures.

5. The failure to show any influence of the surrounding tonic conditions on such dissociation.

An adequate theory must explain all the facts, without exception, and as yet the number of facts determined in this connection are too limited to establish the entire competence of this theory. As a working basis, however, this theory fits all the facts in regard to dissociation that have so far appeared.

On the practical side, by the application of the experimental work set forth in the preceding pages, we have been able to produce sterile extracts of the pneumococcic protective antibody approximating in antibody content the best immune sera, and containing so low a serum protein content that 5 cc. amounts sensitize guinea pigs only irregularly to subsequent injections of horse serum.

The clinical application of these extracts is as yet too limited to warrant drawing adequate conclusions, but the results may be stated to be encouraging and will be the subject of a subsequent report.

ANTIBODY STUDIES

III. CHEMICAL NATURE OF ANTIBODY

F. M. HUNTOON, P. MASUCCI AND E. HANNUM

From the Mulford Biological Laboratories

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The chemical nature of antibodies can be definitely determined only by direct tests on the pure product. Unfortunately, these substances occur in such small proportions that hitherto it has been impossible to isolate them in pure form. Whether the substances involved in immunity reactions are colloids or not, we know that antibodies are closely associated with proteins, and that not only antibody, but some proteins are taken up by antigen in the process of sensitization. Both of these serum constituents may be dissociated during the final extraction of a sensitized antigen. That such a possibility exists will be shown in protocol 4.

Again, the question of the delicacy of the chemical tests at our disposal and the competence of any chemical reaction to detect the presence of antibody as a chemical entity must be considered. The fundamental fact to be borne in mind is that these bodies are comparatively unstable and are easily altered by strong chemical reagents.

We have reason to believe that the chemical concentration of antibodies in the extracts we have employed is, at the least, in a dilution of 1:100,000. This concentration could hardly be detected chemically. It is therefore evident that ordinary chemical methods are of very limited value.

Much information, however, may be gained by indirect chemical and biological methods, and by reactions analogous to those of known chemical substances.

The first question to determine in such a complex problem, is whether antibodies are similar chemically to any of the known

serum proteins or whether they are of a non-protein nature. It has long been known that antibodies are apparently associated with certain serum fractions obtained by salting out methods, and the impression prevails that antibodies are probably of a globulin nature. The salting out methods, however, although useful from a practical standpoint, are of little value in determining the true nature of antibodies owing to the adsorption of the latter by serum proteins.

Ledingham (20) observed that the titer of a diphtheria antitoxin serum could be correlated with the globulin titer. Langstein and Mayer (21) showed that in infected animals an increase of globulin occurred and a decrease of albumin.

However, Glaesner (22) noted that where the immunization of animals was carefully carried out with small quantities of antigen that the globulins did not increase, and that such increase was not necessary for the production of immune bodies.

Nishida and Petroff (23), working with the complement fixing bodies in anti-tubercular sera, came to the conclusion that antibodies are either *part* of the globulins or are carried down by them.

The most that we are justified in stating is that antibodies are carried down by certain precipitates, which, when dissolved, show some of the characteristic properties of such protein bodies.

CHEMICAL TESTS ON SOLUTIONS CONTAINING ANTIBODY

Landsteiner and Jagic (13), working with extracts of hemagglutinins from sensitized red blood cells, concentrated such material until the agglutinin content equalled the original serum, and found the albumin content to be one thirty-third that of the serum.

Von Liebermann and Fenyvessy (18) employed in experiments of this character their concentrated and purified extracts of immune hemolysin. They obtained no positive tests for albumin by the biuret, sulphosalicylic acid, the potassium ferrocyanid or the acetic acid methods, and only traces with the xanthoproteic or Millon's reagent. They also state that only a trace of sulphur could be obtained with alkaline lead solutions and heat.

Kosakai (19) states that, with his solutions of hemolysin, the sulphosalicylic acid tests and the test with salt and acetic acid did not give the same reactions as were obtained with true protein.

Presumably these investigators did not work with pure solutions of antibodies as there is always present a certain amount of impurities derived from the antigen employed. As we have already indicated, it is extremely doubtful that even a pure solution of antibodies could be analysed by the usual protein reagents.

TABLE 1

Saccharose solution

Extract of meningococcus agglutinins. Value = 50,000,000,000 to the cubic centimeter.

Chemical tests of extraction fluid.

Protein color tests:

Biuret—Negative.

Millon's—Negative.

Adamkiewicz—Negative.

Xanthoproteic—Doubtful.

Ninhydrin—Positive.

Precipitation tests:

Heat—No coagulable protein.

Heller's—No precipitate by concentrated nitric acid.

Mereuric chloride—Slight precipitate.

Metaphosphoric—No precipitate.

Picric acid—Abundant flocculent precipitate.

Phosphotungstic—Abundant flocculent precipitate.

Phosphomolybdic—Abundant flocculent precipitate.

Nitrogen content:

Total nitrogen—0.16 mgm. per cubic centimeter.

Amino nitrogen—None.

Some of our chemical tests on antibody extracts are given for the purpose of comparison, and also to show that these tests are not as delicate as the biological methods for determining serum proteins. The extracts tested had a comparatively small amount of serum proteins.

In table 1, is shown the results of chemical tests on an extract containing 50,000,000,000 agglutinins to the cubic centimeter. This extract contained enough bacterial substance to give it a distinct opalescence.

Similar tests on extracts of the pneumococcus protective antibodies are shown in tables 2 and 3.

The results, shown above, indicate that the serum protein content of the extracts tested approaches the minimum amount which can be detected with the chemical reagents at our disposal.

The extract employed in table 3, was also used to determine its ability to produce serum sensitiveness. Five cubic centimeters were injected intraperitoneally into each of two guinea-pigs.

TABLE 2

Extract of pneumococcus protective antibody. Value = 5,000,000 units to the cubic centimeter.

Tentative chemical tests of supernatant fluid.

Color reactions:

Biuret—Negative.

Xanthoproteic—Negative.

Adamkiewicz—Negative.

Ninhydrin—Negative.

Ehrlich's—Negative (dimethylaminobenzaldehyde).

Millon's—Doubtful.

Sulphur—Negative.

Phosphorus—Trace.

Precipitating reagents:

Picric acid—Positive (gives opalescence).

Phosphotungstic acid—Positive (gives opalescence).

Mercuric chloride—Positive (gives opalescence).

TABLE 3

Salt solution extract of pneumococcus protective antibody. Value = 5,000,000 units to the cubic centimeter.

Chemical reactions.

Protein color reactions all negative.

Ninhydrin—Negative.

Sulphur—Negative.

Phosphorus—Negative.

Total Nitrogen—0.035 mgm. per cubic centimeter.

Monoamino acids—None.

Nitrogen in phosphotungstic acid precipitate—0.028 mgm. per cubic centimeter.

After an incubation period of fifteen days, one guinea-pig received an intravenous injection of 1 cc. of normal horse serum. This animal showed a severe anaphylactic reaction, but did not die. The second guinea-pig received an intravenous injection of 1 cc. of antipneumococcic immune horse serum and showed some symptoms but not as severe as the other animal.

This experiment led to the routine testing of our extracts by this method. A summary and discussion of the results will be introduced at a later point.

Our results showed that a considerable number of our extracts would produce serum sensitiveness to subsequent injections of horse serum, and it became necessary to consider the source of the proteins producing such sensitiveness. Were the antibodies responsible or were other serum proteins adsorbed in the process of sensitization?

TABLE 4

Treatment of pneumococcus antigen (sensitizing technic) with immune serum, normal serum and salt solution

Effect on protein content of final dissociation extract.

Sensitization:

150 cc. pneumococcus I antigen added to 50 cc. immune serum.

150 cc. pneumococcus I antigen added to 50 cc. normal serum.

150 cc. pneumococcus I antigen added to 50 cc. salt solution.

Placed at 40°C. for two hours, and then at 5°C. for eighteen hours, after which the mixtures were centrifuged.

Washing: Sediments washed twice with 200 cc. of salt solution.

Dissociation (digestion): Washed sediments, each emulsified in 100 cc. salt solution and placed at 55°C. for thirty minutes. These emulsions were then centrifuged and the supernatant fluids tested as follows:

Chemical and biological tests of final extracts

	IMMUNE SERUM SET	NORMAL SERUM SET	SALT SOLUTION SET
Ninhydrin test	Very faint color. Opalescent	No color. Pro- fuse precipitate	No color. No pre- cipitate
Total nitrogen	0.07 mgm. per cc.	0.08 mgm. per cc.	0.007 mgm. per cc.
Protective antibody content	1,000,000 per cc.	0.0 per cc.	0.0 per cc.

Except in rare cases, the calculated possible dilutions of serum in most of our extracts were too high to warrant expectation of sensitization from this source. Tests made on the fluids employed for wash purposes failed to show any reactions, so that it must be assumed that such results as we obtained were due to proteins present with the antigen until the final dissociation.

An experiment was made to determine whether antigen would take up such substances from normal serum. The nitrogen content of this extract was practically the same as that treated with

TABLE 5
Production of serum sensitiveness by extracts of pneumococcus protective antibody

EXTRACTS PNEUMOCOCCI ANTIBODY	ANTIBODY CONTENT PER CUBIC CENTIMETER	CALCULATED DILUTION OF SERUM PRESENT	SENSITIZ- ING DOSE	ROUTE*	INCUBA- TION PERIOD IN DAYS	INTRA- VENOUS INJECTION OF 1 CC. HORSE SERUM	RESULTS
7 2S	5 million	288 thousand	cc. 5	S	15	Normal	Lived—Slight sneezing
7 2S	5 million	288 thousand	5	P	15	Immune	Lived—Slight sneezing
7 2Y	500 thousand	256 thousand	5	S	15	Normal	Died
7 2Y	500 thousand	256 thousand	5	P	15	Normal	Died
7 2Y	500 thousand	256 thousand	5	P	15	Immune	Lived—sneezed—twitched
7 3G	500 thousand	1 million	5	S	15	Normal	Lived—No symptoms
7 3G	500 thousand	1 million	5	P	15	Immune	Lived—No symptoms
7 3K(1)†	5 million	20 million	5	P	15	Immune	Lived—No symptoms
7 3K(2)	5 million	6 million	5	P	15	Immune	Died
7 3K(3)	5 million	6 million	5	P	15	Normal	Lived—Sneezed—twitched
7 3N	5 million	?	1	P	15	Immune	Lived—Sneezed—twitched
7 3N	5 million	2 million	5	P	15	Normal	Lived—No symptoms
7 3N	5 million	2 million	5	P	15	Immune	Lived—No symptoms
Washing	5 million	3,600	5	P	15	Immune	Lived—No symptoms
7 3R	5 million	1 million	5	P	15	Normal	Died
7 3S	5 million	180 thousand	5	P	15	Normal	Lived—Slight sneezing
7 3T	5 million	38 million	5	P	15	Normal	Lived—Severe twitchings
7 3U	50 million	500 thousand	5	P	15	Normal	Died
	50 million	500 thousand	5	P	15	Immune	Lived—Severe twitchings
7 3W	5 million	2 million	5	P	15	Normal	Died
7 3Y	500 thousand	500 thousand	5	P	15	Immune	Died
7 3Y	500 thousand	500 thousand	5	P	15	Normal	Lived—Restless

immune serum. Both tests show ten times as much nitrogen as in an extract of the same antigen using salt solution throughout the experiment.

An attempt was also made to determine the nature of the antibody content by employing in the sensitizing tests both normal and immune serum for the final dosage. This was a total failure for no consistent differences appeared between the response to normal and immune serum. In table 5 are seen results of a series of tests to determine the possibility of the production of a serum sensitiveness by the injection of extracts of sensitized antigen.

It must be acknowledged that these tests have failed to demonstrate in one way or another the question of the protein nature of antibodies, but have shown that with the technic employed for sensitization of antigen, washing and dissociation, a certain amount of serum protein appears in the final solution, and this amount is sufficient to sensitize a certain proportion of guinea-pigs to horse serum. By continued purifications and final concentration of such antibody extracts, we hope to determine definitely whether highly potent solutions may be obtained free from those serum proteins which sensitize guinea pigs to subsequent injection of serum.

EXPERIMENTS BEARING ON THE NATURE OF ANTIBODIES

The fact that direct chemical tests are not yet available to determine the chemical nature of antibodies, and that it is doubtful whether solutions can be prepared totally free from all other serum constituents and extractive substances derived from the antigen used, necessitates the application of indirect physical, chemical and biological methods in order to throw some light on the problem.

In order to determine whether an extract of antibodies is colloidal, it must be tested by dialysis, diffusion or ultra-filtration. Of the three, the simplest one, at our disposal, is dialysis. Although this method yields purely qualitative results, it gives also an indication of the size of the particle involved; in other words, it tells us whether or not the substance is colloidal in nature.

Kosakai (19) had not been able to dialyse solutions of hemolysin. Some previous work by one of the authors indicated that agglutinins also were not dialysable.

Nishida and Petroff (23) were not able to show dialysis of complement fixing bodies through a celloidin bag.

Using a parchment bag as a dialysing medium, we were able to dialyse a protective antibody solution over a considerable period of time without much loss (protocol 11, section 2).

The results of another dialysing experiment is shown in table 6, in which an extract containing sodium chlorid and sodium bicarbonate was dialysed in fish bladder bags against running tap water.

TABLE 6

Dialysation of salt solution extract of pneumococcus protective antibody

Fish bladder dialysing bags used.

480 cc. placed for 20 hours in running tap water.

Sodium chloride—Before dialysation 0.85 per cent.

Sodium chloride—After dialysation 0.0008 per cent.

Antibody value before dialysation—500,000 per cubic centimeter.

Antibody value after dialysation—500,000 per cubic centimeter.

In another experiment, in which larger quantities were employed, there was apparently some loss but when the precipitate which formed during the process was removed and reemulsified, it was found that a portion of the antibody content had been carried down with the precipitate in sufficient amount to account for the loss. The formation of this precipitate during dialysation is a constant factor, being more marked when salt solution and sodium bicarbonate are used for the final dissociation than when distilled water is so used.

It can be stated, however, that protective antibodies will remain in solution when the sodium chlorid content is one one-thousandth that of physiological salt solution and that little or none is lost through dialysis. Assuming that these antibodies have not been adsorbed by protein impurities present, an assumption which is probable, these experiments point to the conclusion that antibodies have a large molecule, ranging from 0.1 micron to 1 micron in size. In other words, we are dealing with colloidal solutions. Evidence that this colloidal behavior is not due to the

protein impurities present but to the antibodies themselves, is very strong. During dialysis, as already noted, certain proteins, derived either from serum or the antigen, are precipitated out of solution, leaving behind the bulk of the antibodies. Were the antibodies combined with these proteins, we should expect to find them largely in the precipitate, which is not the case. We are, therefore, forced to conclude that antibodies do not dialyse on account of the large size of their molecule.

EFFECT OF TRYPSIN ON PROTECTIVE ANTIBODIES

Dialysis experiments indicate that antibodies are of a complex nature composed of large molecules. Assuming that they are protein in nature and approaching in chemical composition globulins, we should expect on digesting with trypsin the breaking down of the complex molecule into its individual amino acids, with consequent destruction of the antibodies as shown by protection tests.

Experiments were carried out to demonstrate this. As shown in table 7, an excess of trypsin failed to show any reduction in antibody content. Two other experiments in which larger amounts of trypsin were used and in which the time of digestion was one week, yielded identical results (tables 8 and 9).

We made sure that the trypsin was active by running suitable controls both on casein and the extract. When the acid-alcohol precipitating agent was added, there was hardly any precipitate in any of the tubes containing trypsin as compared with the control. The enzyme had apparently digested the serum proteins present, but had not attacked the antibodies.

Besides shedding light on the nature of antibodies, these experiments reveal the rather remarkable resistance that such an antibody-bearing extract offers to the deteriorating influence of a temperature of 37.5°C., and also to such an active enzyme as trypsin.

The fact that trypsin fails to digest antibodies is very significant. We are either dealing with a non-protein substance which cannot be broken into di- tri- and mono-peptides and ultimately amino acids, or, if we are dealing with a protein substance, its composi-

TABLE 7
Effect of trypsin on antibody
 Antibody extract

Known content:

- 0.85 per cent sodium chlorid.
- 0.5 per cent sodium bicarbonate.
- Protective antibody.
- Agglutinins.
- Extracted bacterial substance.
- 0.02 mgm. of nitrogen per cubic centimeter.

Two sets prepared:

- (1) Control, consisting of 15 cc. extract to which 0.25 cc. toluene was added as preservative.
 - (2) Trypsinized, consisting of 15 cc. extract plus 0.25 cc. 1-10,000 dilution trypsin plus 0.25 cc. toluene.
- Both sets placed at 37°C. for 18 hours.

Tests for presence of antibody

Agglutination reaction against pneumococcus type I

	DILUTION			
	1:2	1:3	1:4	Control
1. Control extract.....	4	4	—	—
2. Trypsinized extract.....	4	4	—	—

Protection tests against type I *Pneumococcus*

TEST FLUID	DOSE	PNEUMOCOCCUS CULTURE	MICE
	cc.	cc.	
1. Control extract.....	0.2	0.001	S S
	0.2	0.01	S 52
	0.2	0.04	S 49
2. Trypsinized extract.....	0.2	0.001	S 81
	0.2	0.01	S S
	0.2	0.04	S 42
Virulence control.....		0.00000001	S S
		0.00000001	75 42
		0.0000001	42 28

Tests to establish activity of trypsin. (The solution of trypsin employed was of such strength that 0.23 cc. digested 5 mgm. of casein.)

Added to each extract 5 volumes of 4 per cent acetic acid in 95 per cent alcohol.

1. Control extract. Immediate opalescence with a final flocculent precipitate.

2. Trypsinized extract. No opalescence—no precipitate.

As calculated from the nitrogen content of the extract (0.02 mgm. per cubic centimeter) 2.5 times the necessary amount of trypsin was employed.

TABLE 8
Effect of trypsin on antibody

	PNEUMOCOCCUS PROTECTIVE ANTIBODY EXTRACT	TRYPSIN DILUTION, 1:10,000	SALT SOLUTION	TIME OF INCUBATION AT 37°C.
	cc.	cc.	cc.	
1	5	0.0	0.5	16 hours control
2	5	0.0	0.5	40 hours control
3	5	0.1	0.4	16 hours control
4	5	0.1	0.4	40 hours control
5	5	0.5	0.0	16 hours control

Note. Antibody extract contained 0.25 per cent sodium bicarbonate; reaction, alkaline to phenolphthalein.

Protection tests

EXTRACT	AMOUNT	VIRULENT PNEUMO- COCCUS	AMOUNT	MICE
	cc.		cc.	
1. (16 hour) Control.....	0.2	Type I	0.001	S
	0.2	Type I	0.01	S
	0.2	Type I	0.04	57
2. (40 hour) Control.....	0.2	Type I	0.001	S
	0.2	Type I	0.01	S
	0.2	Type I	0.04	S
3. (16 hour) Trypsinized.....	0.2	Type I	0.001	S
	0.2	Type I	0.01	S
	0.2	Type I	0.04	S
4. (40 hour) Trypsinized.....	0.2	Type I	0.001	S
	0.2	Type I	0.01	S
	0.2	Type I	0.04	S
5. (16 hour) Trypsinized 5 × "3"	0.2	Type I	0.001	S
	0.2	Type I	0.01	93
	0.2	Type I	0.04	S
Virulence control.....		Type I	0.000000001	S
		Type I	0.00000001	57
		Type I	0.0000001	57

tion is such that trypsin does not attack it. The ability to be digested by the enzyme trypsin depends, of course, on the several properties of the molecule. As is well-known, peptides having

the carboxyl-amino linkages (NH-CO) are not split by trypsin. Again, Dakin found that casein and other proteins, which have

TABLE 9

Effect of trypsin on antibody. Continued digestion

Antibody extract

Known content:

0.85 per cent sodium chlorid.

0.4 per cent sodium bicarbonate.

Protective antibody. Three solutions prepared.

A. 100 cc. extract.

0.2 cc. of 1:100 dilution of trypsin.

B. 100 cc. extract—antibody control.

C. 100 cc. salt solution—trypsin control.

0.2 cc. of 1:100 dilution of trypsin.

The three solutions were placed in the incubator and samples removed from each lot at intervals for testing.

Tests for protective antibody content

INCUBATION PERIOD	A			B			C		
	Trypsin-ized extract	Pneumococcus	Mice	Anti-body control	Pneumococcus	Mice	Trypsin control	Pneumococcus	Mice
hours	cc.	cc.		cc.	cc.		cc.	cc.	
24	0.2	0.001	S S	0.2	0.001	S S	0.2	0.00000001	28
		0.01	S 40		0.01	S S		0.00000001	27
		0.04	S 70		0.04	S 70			
48	0.2	0.001	S 41	0.2	0.001	S S			
		0.01	S 88		0.01	S S			
		0.04	S 27		0.04	S 88			
168	0.2	0.001	S	0.2	0.001	S			
		0.01	52		0.01	S			
		0.04	18		0.04	90			
Virulence control		0.00000001	S 42						
		0.00000001	40 28						

It will be noted that the results with "C" trypsin control show that no protective action can be attributed to trypsin as such.

been racemized by dilute alkali, are no longer digested by trypsin. We have, therefore, these two facts to work upon. As this is merely a preliminary report on the chemical nature of antibodies,

further work is being done to determine more definitely whether we are dealing with a complex peptide containing the carboxyl-amino group or whether the antibodies have been racemized by dilute alkali, which in either case resists the action of trypsin.

A consideration of protocols 21 and 23 in the preceding section, shows that the presence of mild alkalis does not injure the anti-

TABLE 10

Purification of antibody extract; effect of acidification on the antibody content

To 50 cc. of extract was added 0.5 cc. of glacial acetic acid—flocculation occurred.

The mixture was placed on ice over night. It was then centrifuged and the clear supernatant fluid treated as follows: To 10 cc. was added 0.05 cc. acetic acid—no turbidity apparent. The remainder was neutralized to phenolphthalein with N/1 NaOH solution, and designated as solution A.

The sediment was then shaken up in 5 cc. of salt solution and thus concentrated 10 times. This was designated as solution B.

Protection tests

TEST FLUID	DOSE	PNEUMOCOCCUS	MICE
	cc.	cc.	
Original material.....	0.2	{ 0.001 0.01 0.04	S S 23
A. Acetic acid supernatant fluid.....	0.2	{ 0.001 0.01 0.04	S S 16
B. Emulsified sediment (concentrated 10 times)...	0.2	{ 0.001 0.01 0.04	S S 42
Virulence control.....	{	0.00000001 0.0000001 0.0000001	S 42 28

body, but renders it filterable, perhaps by reversing the electrical charge.

The following experiment, shown in table 10, demonstrates that no great injury to the antibody takes place in the presence of mild acids such as acetic acid.

An extract of protective antibody showed on the addition of acetic acid some turbidity, indicating the presence of protein

substances. The experiment was made in an endeavor to purify such an extract.

The results of this experiment show that a certain amount of antibody was adsorbed by the precipitate formed, but that the bulk remained uninjured and in solution.

The following is a summary of our present knowledge as to the chemical nature of antibody:

1. The antibody molecules are of large size, not being dialysable, indicating the colloidal nature of the substance.

2. Antibodies are not affected by trypsin over considerable periods indicating either that they are not protein in nature, or have been racemized by the dilute alkali used, or belong to the peptide group having a carboxyl-amino linkage.

3. Antibodies are not precipitated by solutions containing little or no electrolyte content, indicating that they are not of a euglobulin nature.

4. Antibodies are not soluble in ether, therefore are not of the lipin group.

5. Antibodies free from any gross amount of globulins are not precipitated or affected by a short exposure to 30 per cent sodium chlorid solution, indicating that they are not of a pseudoglobulin nature.

6. Antibodies are not injured by certain dilute alkalis or acids.

7. Antibodies are not affected by temperature up to 60°C. Higher temperatures progressively destroy or alter their nature.

We may state therefore that antibodies do not belong to that group of proteins usually considered under the head of serum proteins.

It is felt that in the present state of knowledge of protein chemistry, the negative information such as we have obtained by indirect methods is of value in narrowing the possible field of investigation.

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A NOTE ON THE CLAIM THAT AGGLUTININS ARE LIPOIDAL IN NATURE

CHARLES KRUMWIEDE AND W. CAREY NOBLE

From the Bureau of Laboratories, Department of Health, City of New York

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The claim that agglutinins could be extracted from sera by the use of lipoidal solvents, was called to our attention by the address of Jobling (1) on the relation of lipoids to immunity. This claim was made by Stuber (2), (3) who believed he had demonstrated that he could extract the agglutinins from an immune serum with petroleum ether and that the addition of the extracted substances to normal serum would result in an agglutinating value practically equal to that of the unextracted immune serum. He claimed, also, that the serum of normal animals became strongly agglutinative after the animals had received intravenous injections of such extracted substances.

We have attempted to duplicate the results of Stuber without success. However, we hesitated about presenting our findings, first, because of their negative character and secondly, because of a later article by Stuber (3) on the same subject. If we understand him aright, the statements in regard to the activity of serums after minimal additions of the extracted substances are so extraordinary as to induce a strongly skeptical attitude towards all his results. This skepticism would seem to be justified by the fact that the agglutinins are precipitated with the globulins of the serum. It must be admitted, however, that the possibility that agglutinins are lipoidal in nature is not excluded, as they may be carried down with the globulins through the mechanism of adsorption. Because his conclusion that agglutinins resemble lipoids is so contrary to our ideas and as his results have attracted attention it was finally decided to record very briefly our negative findings.

In his earlier report (2) Stuber mentioned merely ether as the solvent extractive. This led us to use ethylic ether for our earlier experiments. The later experiments were carried out with petroleum ether. Stuber states that he extracted his serum in a "Lindschen Apparat." We have been unable to obtain information as to the nature of this apparatus and have therefore not duplicated his procedure in this detail. However, the methods we have employed should be sufficiently effective to extract agglutinins, if they are soluble in lipoidal solvents. Representative experiments are as follows. All agglutinations were carried out with the Dreyer antigen and readings were made after incubation at 56°C. for two hours. A complete reaction is indicated by ++, lesser reactions by the symbols +1, +, ± and negative by -.

Experiment I. To 10 cc. of anti-typhoid serum (horse) 50 cc. of ether were added and the mixture was shaken for fifteen minutes. The ether was separated and collected; to the residual serum 25 cc. more of ether were added. The mixture was again shaken, the ether separated and collected. These ether extracts were combined and added to 10 cc. of normal horse serum. The ether was driven off from this mixture and also from the extracted immune serum by evaporation at 45°C.

Agglutination results

Anti-typhoid serum—not extracted.....	1:40,000	++
Anti-typhoid serum—extracted with ether.....	1:40,000	+1
Normal serum plus ether extract*.....	1: 2,000	+1
Normal serum.....	1:10	-

* When a normal serum was similarly extracted, and the ether extract added to another normal serum and the ether evaporated as above, no agglutinating properties were conferred on the second normal serum. This experiment was tried to exclude the possibility of agglutination due to physical change in the serum.

Experiment II.—Beef serum was extracted with ether as in experiment 1. The ether extract was evaporated to dryness and the residue taken up in 5 cc. of saline. After clarification by centrifuge, this residue as well as the extracted serum and a sample of unextracted beef-serum were utilized as antigens in precipitation tests with rabbit anti-beef serum. The dilutions given are those of the antigens.

Precipitation results

Ether extract in saline.....	1:10	+
Beef serum after extraction.....	1:1000	+
Beef serum not extracted.....	1:2000	+

In experiment I there is an apparent transfer by the ether of some agglutinins. This apparent transfer was not due to a physical change in the serum to which the ether extract had been added. Experiment II indicates clearly that the apparent transfer was due to carrying over the serum proteins as a fine emulsion of serum in the ether and possibly also through adsorption by the lipid solution.

Stuber refers to a further enhanced action by the serum to which ether extractives have been added, if the mixture is incubated at 37°C. for one hour. He attributes this additional activity to solution of the lipoids by action of the serum lipase. We tested this point as follows. Serums to which the ether extractives had been added were incubated at 37°C. for one, two, three and eighteen hours respectively and then tested. The agglutination results showed no change when compared with tests carried out before incubation of the mixture.

A repetition of the above experiments carried out with petroleum ether gave such similar results that a description of the details would be needless.

A final test of the question under discussion would evidently be the extraction of finely divided dried serum. Anti-typhoid serum (horse) was dried at 46°C. with the aid of an electric fan, ground as fine as possible in a mortar and held at 37°C. till constant in weight and then weighed. At the suggestion of Dr. John Mandel only the lower boiling point fractions of petroleum ether were employed for extraction. These were obtained by distilling at 45°C.

Experiment III.—Separate samples of the dried serum, 2.5 grams, were extracted with 50 cc. of petroleum ether, 50 cc. of ethylic ether and 50 cc. of water respectively at 25°C. for eighteen hours. For the first few hours the samples were shaken frequently. After centrifuging, 25 cc. of the supernatant fluid from each of the first two samples were added to 25 cc. of salt solution. These and the water extract control

were heated at 45°C. till the solvents were completely evaporated. Amounts of 0.25 cc., 1.0 cc., and 5.0 cc. were added to separate 10.0 cc. samples of normal horse serum, thoroughly shaken and incubated at 37°C. for two hours. The mixtures and watery extract controls were then tested for their agglutinin content.

Agglutination results

Normal serum plus ether extractives in salt solution

Serum 10 cc. plus 0.25 cc.....	1:10 negative
Serum 10 cc. plus 1 cc.....	1:10 negative
Serum 10 cc. plus 5 cc.....	1:10 negative

Normal serum plus petroleum ether extractives in salt solution

Serum 10.0 cc. plus 0.25 cc.....	1:10 negative
Serum 10.0 cc. plus 1 cc.....	1:10 negative
Serum 10.0 cc. plus 5 cc.....	1:10 negative

Normal serum plus water extract

Serum 10.0 cc. plus 0.25 cc.....	1:800	+
Serum 10.0 cc. plus 5 cc.....	1:10,000	+

CONCLUSIONS

There is nothing in the above experiments to indicate that agglutinins are lipoidal in character to the extent that they are soluble in lipid solvents. In no case were we able to reduce the titer of extracted sera beyond a reduction which is explainable by the manipulation of the serum. In no case was there a transfer of agglutinins by the extracting solvent where we could not at the same time demonstrate a transfer of antigenic proteins. The methods employed were relatively simple. This was desirable in view of the destruction of the antibody by more complex methods. However, the methods used would suffice to demonstrate the solubility of agglutinins in the solvents used, if they were soluble in these menstrua.

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THE RELATION BETWEEN THE ABSORPTION OF ANTIBODIES AND THE ISOLATED PROTEIN BODIES

ROKURO UMEMURA

From the Serological Laboratory of the Tokyo Institute for Infectious Diseases

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In the production of serum for therapeutic purposes, a most important question to be considered, from the practical standpoint, is how to eliminate its therapeutically useless protein bodies and to leave, at the same time, the maximum of antibodies in the minimum quantity of dose. Pick (9), Porges and Spiro (10), Gibson (3), and, Banzhaf and Gibson (1) have all studied this problem with the view of determining just which serum protein contains the various antibodies and they have separated the antibodies in part or in whole; furthermore, Banzhaf and Gibson have made practical applications of these results and today their methods are widely used in Europe and especially in the United States. But, from the researches on diphtheria antitoxin by Madsen (5, 6), Henderson-Smith (4), and others, it appears that the concentrated antibodies are only very slowly absorbed.¹ Again, Walbum (11) distributed the serum into four strata by a freezing method and made a special study of the stratum which contained the maximum quantity of antibodies, and attributed its slow absorption to the presence of a large amount of proteins. Whether or not there is any relation, with respect to the speed of their absorption, between

¹ The only significant study with conflicting results is that by Park, Famulener and Banzhaf (9) who, after testing the effect of protein concentration upon the absorption of an immune horse serum injected subcutaneously into goats, normal men and rabbits, conclude that "the degree of protein concentration which is usually employed to produce the refined and concentrated diphtheria antitoxic globulin preparations has little or no effect in retarding the absorption of the antitoxin from the subcutaneous tissues."

the antibodies and pseudoglobulin which is easily soluble in water, on the one hand, and euglobulin, which is not easily soluble in water, on the other, is an interesting question from practical and theoretical points of view. As it has been demonstrated by Henderson-Smith and others that diphtheria antitoxin and other antibodies are of the same general nature, I have attempted to study the problem with the agglutinin of the serum of a horse immunized against the typhoid bacillus.

NATURE OF ISOLATED PROTEIN BODIES

Following the related researches of previous investigators, Marcus (7) established a distinction between the water-soluble and water-insoluble protein bodies. Hofmeister and Pick (9) discovered the fact that water-insoluble protein bodies can be precipitated at 2.8–3.6 saturation of ammonium sulphate, while water-soluble proteins came down at 3.6–4.4 saturation. The former they called euglobulin and the latter pseudoglobulin. According to Porges and Spiro (10), there are three varieties of serum proteins; the first is precipitated at 2.8–3.6 saturation, the second at 3.3–4.2 saturation, and the third at 4.0–4.6 saturation of ammonium sulphate, and each contains a soluble protein. Furthermore, in line with the discovery of Freund and Joachim (2), using the method of Pick, that both euglobulin and pseudoglobulin contain not only soluble but also insoluble protein bodies, I have observed the fact that, in the process of dialysis of the precipitates from the serum at one-third and one-half saturations with purified ammonium sulphate, both were completely soluble during the first day or two when the salt was still present, while, after three or four days, pseudoglobulin was almost completely dissolved in water, leaving only a small amount of precipitate, and euglobulin remained undissolved for the most part, although some had evidently gone into solution. I have, therefore, prepared my proteins as follows:

To 500 cc. of the horse serum immunized against the typhoid bacillus, with an agglutination titer of 1:60,000, 2000 cc. of distilled water is added and, while stirring, 1250 cc. of saturated

aqueous solution of ammonium sulphate is gradually added. The precipitate thus formed at 3.3 saturation is filtered after three hours, and the process repeated until the filtrate becomes absolutely clear. The filtrate is again half saturated with ammonium sulphate and the precipitate is filtered repeatedly until the filtrate is clear. Both precipitates are then washed with 3.3 and 5.0 saturated solutions of ammonium sulphate, respectively, repeating the process until the washings give a negative result when tested with Spiegler's solution. When the test for sulphate in the precipitate becomes negative to barium chloride and the ammonia with Nessler's reagent, the greater amount of the first precipitate is dissolved in physiological saline which furnishes us with a turbid solution of euglobulin, while the greater part of the second precipitate is dissolved in water which gives us a bluish transparent solution of pseudoglobulin.

ABSORPTION EXPERIMENTS

In carrying out the present experiments, it is absolutely essential that we pay particular attention to the normal agglutination value in the serums of the experimental animals. Thus we selected only the rabbits whose normal serum, after repeated determinations, was found to possess a normal agglutination titer for the typhoid bacillus of not more than 1:40. Each protein body, which was prepared according to the methods described in the preceding paragraph, was diluted with distilled water until it showed the same agglutination titer as that of the undiluted serum of a horse immunized against typhoid bacillus (1:20,480). Moreover, the amount of protein in each was determined with the use of the Kjeldahl method, as shown in table 1. Ten cubic centimeters of each were then injected into the various tissues of the rabbits, referred to in the tables, and at successive intervals specimens of serum were obtained by bleeding from the auricular vein. These specimens were stored in the ice box until the last one had been obtained, when all were tested.

In the agglutination test each specimen of serum was diluted progressively with sterile physiological saline. The bacillary

suspension was made by emulsifying in 10 cc. of sterile physiological saline the eighteen-hour growth of a typhoid culture on an agar slant. The agglutination reaction was determined by adding two drops of the bacillary emulsion to the serum dilutions and incubating for two hours at 37°C. and then for twenty-four hours at room temperature.

TABLE 1

DILUTION	SERUM 7.52 PER CENT	EUGLOB- ULIN 2.56 PER CENT	PSEUDO- GLOBULIN 7.79 PER CENT	DILUTION	SERUM 7.52 PER CENT	EUGLOB- ULIN 2.56 PER CENT	PSEUDO- GLOBULIN 7.79 PER CENT
1:40	+++	k	3	1:2560	+++	k	3
1:80	+++	k	3	1:3600	+++	k	3
1:160	+++	k	3	1:5120	+++	k	3
1:320	+++	k	3	1:7200	+++	k	3
1:640	+++	k	3	1:10,240	+++	k	3
1:900	+++	k	3	1:14,400	++	d	2
1:1280	+++	k	3	1:20,480	+	s	1
1:1500	+++	k	3				

Note: In this and all subsequent tables, the use of the same signs is observed with the following signification:

+++ , ++ , + are applied to the serum in the order of strong, medium and weak reactions.

k, d, s, are applied to euglobulin in the order of strong, medium and weak reactions.

3, 2, 1, are applied to pseudoglobulin in the order of strong, medium and weak reactions.

1. Subcutaneous injections

Subcutaneous injections were made as follows: Rabbit 1 (weight, 2100 grams) injected with 10 cmm. of the euglobulin preparation. Rabbit 2 (weight, 2000 grams) injected with 10 cmm. of the pseudoglobulin preparation. Rabbit 3 (weight, 2150 grams) injected with 10 cmm. of the whole serum.

The results of examination at stated intervals are shown in table 2.

From the above table it is plain that euglobulin, even at the end of the first hour, shows a double amount of absorption and reaches the maximum degree in seventy-two hours. On the contrary, in spite of the fact that pseudoglobulin had been

absorbed in the same degree as the whole serum during the first twelve hours, it drops below the serum after twenty-four hours, attaining its maximum degree only at the end of four days.

2. Intramuscular injections

The intramuscular injections were made in the interscapular region as follows:

Rabbit 4 (weight, 2000 grams) injected with the euglobulin preparation.

Rabbit 5 (weight, 2200 grams) injected with the pseudoglobulin preparation.

Rabbit 6 (weight, 2100 grams) injected with the whole serum.

The degree of absorption is shown in table 3.

According to the above table, it is evident that within the first thirty minutes there appears a marked difference between the three substances. But, while euglobulin attains its maximum reaction at the end of six hours, both the whole serum and pseudoglobulin take twenty-four hours to attain their maximum degree of agglutination.

3. Intravenous injection

In this series of experiments, the substances were injected into the left auricular veins and the serum was obtained from the right at stated intervals. The injections were made as follows:

Rabbit 7 (weight, 1950 grams) injected with the euglobulin.

Rabbit 8 (weight, 2050 grams) injected with the pseudoglobulin.

Rabbit 9 (weight, 1990 grams) injected with the whole serum.

The decrease in the agglutination values are detailed in table 4.

From the above table it is evident that between euglobulin and the whole serum there is very little difference in the rate of decrease in the agglutination values, but the decrease in the case of pseudoglobulin is markedly retarded.

TABLE 2

INTERVALS	INJECTION	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:900	1:1280	1:1800	1:2560	1:3600	1:5120	1:7200
1 hour.....	S E P	+++ k 3 3	+++ k 3 3	+++ k 2 1	+	s									
3 hours.....	S E P	+++ k 3 3	+++ k 3 3	+++ k 2 1	+	d 1	s								
6 hours.....	S E P	+++ k 3 3	+++ k 3 3	+++ k 3 2	+	k 2	d 1	s							
12 hours.....	S E P	+++ k 3 3	+++ k 3 3	+++ k 3 3	+++ k 3 3	+++ k 3	+++ k 2	+	s						
24 hours.....	S E P	+++ k 3 3	+++ k 3 3	+++ k 3 3	+++ k 3 3	+++ k 3	+++ k 3	+++ k 2	+++ k 1	+	s				
3 days.....	S E P	+++ k 3 3	+++ k 3 3	+++ k 3 3	+++ k 3 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 2	+++ k 1	+++ d	+	s	
4 days.....	S E P	+++ k 3 3	+++ k 3 3	+++ k 3 3	+++ k 3 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ d 2	+	s	1	

[illegible]

Abbreviations: S = serum; E = euglobulin; P = pseudoglobulin. These abbreviations are used in all subsequent tables.

TABLE 3

INTERVALS	INJECTION	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:900	1:1280	1:1800	1:2560	1:3600	1:5120
30 minutes.....	{ S E P	++ k 3	++ k 3	++ k 2	++ k 1	++ d	s							
1 hour.....	{ S E P	++ k 3	++ k 3	++ k 3	++ k 2	++ k 1	++ d	s						
3 hours.....	{ S E P	++ k 3	++ k 3	++ k 3	++ k 3	++ k 2	++ k 1	++ k	d	s				
6 hours.....	{ S E P	++ k 3	++ k 3	++ k 3	++ k 3	++ k 3	++ k 2	++ k 1	++ k	++ k	d	s		
12 hours.....	{ S E P	++ k 3	++ k 3	++ k 3	++ k 3	++ k 3	++ k 3	++ k 3	++ k 2	++ k 1	++ d	s		
24 hours.....	{ S E P	++ k 3	++ k 3	++ k 3	++ k 3	++ k 3	++ k 3	++ k 3	++ k 3	++ k 3	++ d	++ s	++ 1	
3 days.....	{ S E P	++ k 3	++ k 3	++ k 3	++ k 3	++ k 3	++ k 3	++ k 3	++ k 3	++ k 3	++ d	++ s	++ 1	

[illegible]

4. Intraperitoneal injection

From the standpoint of practice, there is no apparent bearing in the absorption of antibodies from the peritoneal cavity, but it is of interest from the standpoint of theory. The injections were made as follows:

Rabbit 10 (weight 2100 grams) injected with the euglobulin.

Rabbit 11 (weight, 2050 grams) injected with the pseudoglobulin.

Rabbit 12 (weight, 1950 grams) injected with the whole serum.

The result of examinations are shown in table 5.

In the above experiments, also, euglobulin was the first to attain its maximum degree of absorption, while the whole serum reached its maximum at the end of the sixth, and the pseudoglobulin at the end of the twelfth hour.

ABSORPTION POWER AND QUANTITY OF PROTEINS

In the preceeding experiments we have discovered the fact that the absorption of euglobulin was first in order, followed by the unaltered serum and the pseudoglobulin. But, as indicated in table 1, there is a great difference in the quantity of proteins present in the euglobulin preparation and the whole unaltered serum, and the speed of absorption in the case of euglobulin may be due to a lesser quantity of proteins contained in it. In order to decide this point, another set of experiments was performed in which agglutination values were determined with the euglobulin from the immune serum to which is added euglobulin obtained in a similar manner from normal horse serum up to the percentage of the protein content of the whole immune serum and also to 15 per cent. The intraperitoneal injections were made as follows:

Rabbit 13 (weight, 1970 grams) injected with 7.66 per cent euglobulin.

Rabbit 14 (weight, 2200 grams) injected with 15 per cent euglobulin.

The results are as follows (table 6).

Interpreting the above results, we may state that the tardiness in the absorption of isolated protein bodies is due not only to the fact that they are denatured but also to the quantity of proteins contained in such serum preparations. This is evident from the fact that the euglobulin, which showed a greater speed in absorption than the normal serum, became decidedly tardy when protein was added to equal the quantity found in the normal serum, and when this quantity was doubled, the absorption was not only retarded but attained to only one half of the absorption value of the normal serum.

SUMMARY AND CONCLUSIONS

The absorption of antibodies obtained together with the isolated proteins, in harmony with the results of investigations by Th. Madsen, J. Henderson-Smith, and Walbum, depends upon the quantity of proteins associated with them. Thus Kraus, in his experiments on absorption of antibodies in normal serum, showed that the maximum degree was attained in fifty-three hours after subcutaneous injection, while it was reached after intraperitoneal injection in thirty hours. But Walbum differs from Kraus in claiming that it took forty hours after intraperitoneal injection to attain the maximum of absorption. This diversity of results is largely due to the difference in the quantity of proteins contained in the serums which they used; but, when the quantity of proteins contained in the material is the same, the absorption of the normal whole serum is faster than that of the isolated proteins. Thus in the practical application of antibodies, it is useless to try to increase the antibody values by means of sheer concentration, without taking into account the quantity of proteins contained in the preparation, if one is to work for the maximum speed of absorption. Hence, we insist on the use of antibodies completely isolated by means of ammonium sulphate, which are associated with comparatively small quantities of proteins. We may draw the following conclusions:

1. The rate of absorption of antibody depends upon the quantity of proteins associated with it.

TABLE 5

INTERVALS	INJECTION	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:900	1:1280	1:1800	1:2560	1:3600	1:5120	1:7200	1:10,240
5 minutes.....	S E P	+++ k 3	+++ k 3	+++ k 2	+++ k 1	++ d	s									
15 minutes.....	S E P	+++ k 3	+++ k 3	+++ k 3	+++ k 2	+++ k 1	++ d	s								
30 minutes.....	S E P	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 2	+++ k 1	+++ k	++ d	s						
1 hour.....	S E P	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 2	+++ k 1	+++ k	+++ k	++ d	s				
3 hours.....	S E P	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 2	+++ k 1	+++ k	++ d	s			
6 hours.....	S E P	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 2	+++ k 1	+++ d	++ s		
12 hours.....	S E P	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ k 3	+++ d	++ s	++ 1	

TABLE 6

TIME	FIGURED IN PER CENT		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:900	1:1280	1:1800	1:2560	1:3600	1:5120	1:7300	1:10,240
	{	}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5 minutes....			k	d	s	+											
15 minutes...	{	}	k	k	d	s	+										
30 minutes...			k	k	k	d	s	+	+								
1 hour.....	{	}	k	k	k	k	d	s	+	+							
3 hours.....			k	k	k	k	k	d	s	+	+	+					
6 hours.....	{	}	k	k	k	k	k	k	d	s	+	+	+				
12 hours.....			k	k	k	k	k	k	k	d	s	+	+	+	+		
24 hours.....	{	}	k	k	k	k	k	k	k	k	d	s	+	+	+		
3 days.....			k	k	k	k	k	k	k	k	k	d	s	+	+		
4 days.....	{	}	k	k	k	k	k	k	k	k	d	s	+	+	+		
			k	k	k	k	k	k	k	k	d	s	+	+	+		

2. The quantity of proteins contained being the same, the rate of absorption of antibody associated with isolated protein bodies is slower than that of the whole normal serum.

3. There is no difference in the rate of absorption of the antibodies of each isolated protein.

4. Hence there is an advantage in preferring isolated protein bodies, containing a minimum of protein, in the use of antibodies.

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SPECIFICITY OF ANTI-ORGAN SERA

MOYER S. FLEISHER AND NATALIE ARNSTEIN

From the Department of Pathology and Bacteriology of St. Louis University School of Medicine, St. Louis, Missouri

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In a previous article (1) we have shown that there exist between antisera and antigens of liver or kidney certain distinguishing reactions which apparently indicate the existence of a definite biological difference between these two organs. This difference was demonstrated by either cross fixation or by fixation following absorption of the sera by the respective homologous and various other organ cells. We purposely avoided the use of the term specificity because we considered it essential to extend our investigation to include other organs before we might consider the differences noted as definite evidences of tissue specificity.

We also noted in our experiments that both the antigens and the antisera were apparently rather complex and appeared to contain at least three distinct partial antigens or antibodies. The first of these antigens had apparently a wide range of activity and presumably possessed a relationship to all organs of the species. The second had a limited range of activity and reacted only with the tissue used in the preparation of the antigen or antibody. The third was apparently composed of a group of antibodies, also rather limited in their activity and these reacted only or most strongly with individual tissues other than the one used as the immunizing substance.

We suggested in our earlier article that it would be of interest to determine whether similar definite relationships could be noted in other organs and we wish here to report a series of experiments directed towards determining these facts.

We carried out complement fixation experiments with liver, kidney, spleen, brain, testicle and muscle of guinea-pigs. We

tried cross fixation experiments and also fixation experiments after having absorbed the various sera with different tissues. Our technic was the same as in our earlier experiments and we will not here repeat a description of the methods used (1).

CROSS FIXATION EXPERIMENTS

In these experiments we tested each antiserum against each one of the antigens, determining the titer which gave fixation in each case. There appeared slight variations in the results in individual experiments, possibly in part because we used antisera from different rabbits immunized against the same organs, and also because in the different experiments we used different antigens. It is quite possible that in different antisera prepared against the same organs the relative quantities of the partial antibodies might vary, and it is certain that the individual antigens varied slightly in their antigenic qualities and may possibly have varied in their content of the partial antigens. We attempted however in each series of titrations of a single antiserum or group of antisera to keep constant as many factors as possible. We have determined the mean of the results of all cross fixation experiments in constructing the chart below, since we believe this to be a fairer representation of the results than would be an example of a single experiment or of a number of individual experiments. Furthermore, the results are probably clearer when shown thus graphically.

Considering first the kidney antiserum we note that it fixed in the highest dilution with kidney antigen, next with testicle antigen and spleen antigen, in the order named, and only poorly with liver, brain and muscle antigens.

Liver antiserum gives the best fixation with testicle antigen, and fixes with liver antigen in only slightly smaller quantities. Then in the order named follow kidney, spleen, muscle and brain antigens.

Spleen antiserum fixes best with spleen antigen, gives pretty good fixation with kidney antigen, and fixes less and about equally as well with liver, brain, testicle and muscle antigen.

Brain antiserum fixes best with testicle antigen, nearly as well with brain antigen and distinctly more poorly with the other four antigens.

Testicle antiserum fixes well with all of the antigens but slightly better with testicle antigen than with the rest.

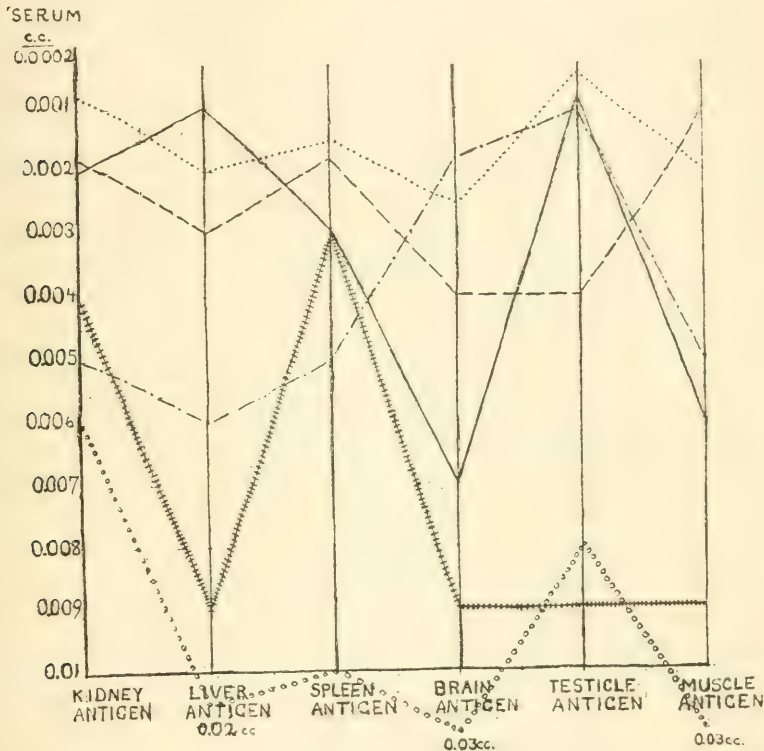


FIG. 1. CROSS FIXATION WITH ANTITISSUE SERA AND TISSUE ANTIGENS

The ordinate shows the mean of the smallest quantity of the serum which gave fixation with the various antigens.

The perpendicular lines represent the various antigens.

The various sera are represented by the continuous or broken lines as follows:

Kidney	Liver ———	Spleen ————
Brain —.—.—.	Testicle	Muscle ————

Muscle antiserum gives the best fixation with its corresponding antigen, only slightly lower titration with spleen and kidney and still poorer with liver, brain and testicle.

We note therefore in the cases of kidney, spleen, testicle and muscle antisera what is apparently a specific relationship between the sera and the corresponding antigens, in other words apparently a reaction which we may well consider as pointing towards a tissue specificity.

In the case both of liver and of brain antiserum we find that fixation is best with testicle antigen, the corresponding antigen being in each case a close second. However, if we compare the fixation of all of the various antisera with these two antigens (brain and liver) we note that the respective homologous antisera give the best fixation. Furthermore, we find that testicle antigen gives excellent fixation with most of the antisera, suggesting that testicle furnishes a particularly good antigen. So that even in the case of liver and brain it seems that there is a relative specificity shown by cross fixation.

It is of interest to inquire further into the relationship of these various antisera and antigens to each other. We find that kidney furnishes a good antigen reacting quite well with practically all the antisera. Kidney antiserum, on the other hand, reacts rather poorly with practically all of the antigens. Testicle also makes a good antigen, reacting quite well with the six antisera, and testicle antiserum gives good fixation with all of the antigens. The other four organs form antigens which are less regular in their activity than these first two; spleen gives possibly a slightly better antigen than liver, brain or muscle but the difference is only slight. On the other hand muscle antiserum reacts well with all the antigens; brain antiserum reacts well only with brain and testicle antigen and relatively poorly with the rest; liver antiserum reacts well with liver and testicle and kidney antigens, and fairly well with the other three, while spleen antiserum reacts well with spleen and kidney antigen and distinctly more weakly with the other antigens.

From these considerations we can possibly draw some preliminary conclusions regarding the value of the different organs as antigens. It appears evident that testicle is the best antigen of the six, since in at least two cases it gives the best fixation with sera not prepared against this organ, in other words, in

non-specific reactions. Kidney, however, is only a little less active and in addition it is rather more regular in its antigenic activity with the various sera. Spleen antigen furnishes about the third best antigen of the organs examined and brain, muscle and liver antigens are the poorest and about equally so.

If we compare the reactions of the various antisera it appears that in general they react in the following order, starting with the best serum; testicle, muscle, liver, brain, spleen and kidney. It is interesting to note the constancy with which the order in which the various sera tend to react with the antigens is maintained; it remains practically the same except when the specific relationship between antigen and antiserum changes it slightly.

The fact, noted above, that testicle antigen is apparently an exceptionally strong antigen would, to a certain extent, influence the interpretation of the results with both brain and liver antisera; we find that the reaction of these antisera is stronger with testicle antigen than it is with the respective specific antigens. Both of these antigens—brain and liver—are apparently relatively poor antigens, and yet when tested with their corresponding specific antisera these two antigens are second only in activity to the strongly antigenic testicle antigen. It seems, therefore, that if we take these facts into consideration we have evidence of tissue specificity, apparently however only relative.

If now we compare the activity of the various organs as antigens and as antibody producing agents, we find that testicle shows marked activity in both of these directions; kidney, however, is a good antigen but the antikidney serum has only weak fixing powers. Muscle, on the other hand, produces a serum of good activity but the muscle antigen is rather weak. Liver, spleen and brain are all about alike in their activities and they produce fair antisera and fair antigens.

We see therefore that antigenic qualities as represented by the power of the organs to react with various antisera, and antibody producing powers as represented by the reaction of the sera with the various antigens, do not always run parallel, and this fact we shall again refer to in later discussion.

It is further interesting to note that both muscle and spleen antiserum are the only ones which react relatively poorly with testicle antigen, which, as stated above, is apparently the strongest antigen; especially is this of interest because of the fact that both spleen and muscle are of mesenchymal origin.

ABSORPTION AND COMPLEMENT FIXATION

We have carried out experiments in which we absorbed the various sera with emulsions of the washed cells of various organs before we tested the fixing power with the various antigens. Concerning the details of the technic we refer to our earlier article (1).

The results of these experiments are shown in the following charts, which represent the mean of several experiments in each case.

We shall consider first only tissue specificity as shown in the reactions between the antiserum and its homologous absorbent. In kidney serum which was tested only with kidney, liver, spleen and brain antigens and absorbents, we find that with kidney antigen the specificity is marked and evident, and with the three other antigens the relationship between kidney and its homologous absorbent is fairly well shown.

Liver antiserum shows quite definitely specific effect of the homologous absorbent with liver, kidney, spleen, brain and muscle antigens; with muscle antigen, however, the relationship is not marked; in the experiments with testicle antigen there was no evidence of any specific relationship between liver serum and its specific absorbent.

Brain antiserum showed specificity with the homologous absorbent when tested with brain, kidney, muscle and testicle antigens but did not show this relation when tested with liver and spleen antigens.

Spleen antiserum did not show very markedly the relationship between the serum and the homologous absorbent. The relationship was suggested however in the reactions with three antigens, spleen, muscle and testicle. In kidney, liver and brain

TABLE 1

Absorption of immune bodies from tissue antisera determined by complement fixation

ABSORBENTS	KIDNEY ANTIGEN	LIVER ANTIGEN	SPLEEN ANTIGEN	BRAIN ANTIGEN	MUSCLE ANTIGEN	TESTICLE ANTIGEN
Antikidney serum						
Kidney	++++	++	++++	++	—	—
Liver	++	+++	++	+++	—	—
Spleen	0	0	+++	?	—	—
Brain	0	0	?	+++	—	—
Muscle	—	—	—	—	—	—
Testicle	—	—	—	—	—	—
Antiliver serum						
Kidney	++	+	++	++	0	0
Liver	++++	++++	++++	++++	+	0
Spleen	0	0	++	+	0	0
Brain	0	0	+	++	0	0
Muscle	0	0	0	0	0	0
Testicle	0	0	0	0	0	0
Antispleen serum						
Kidney	++	?	?	+	?	?
Liver	+	++	?	++	?	?
Spleen	0	0	+	0	+	+
Brain	0	0	0	+	0	0
Muscle	0,	0	0	0	0	0
Testicle	0	0	0	0	0	0
Antibrain serum						
Kidney	++	?	++	?	+	?
Liver	++	+	++	+	+	++
Spleen	+	+	+++	?	0	0
Brain	++++	?	++	++++	++++	++++
Muscle	0	0	0	0	0	0
Testicle	0	0	0	0	0	++
Antimuscle serum						
Kidney	+++	++	+	0	0	0
Liver	0	++++	+	?	0	0
Spleen	0	0	?	0	0	0
Brain	0	0	0	?	0	0
Muscle	0	+++	0	?	0	0
Testicle	0	0	0	0	0	0

TABLE 1—*Continued*

ABSORBENTS	KIDNEY ANTIGEN	LIVER ANTIGEN	SPLEEN ANTIGEN	BRAIN ANTIGEN	MUSCLE ANTIGEN	TESTICLE ANTIGEN
Antitesticle serum						
Kidney	+	0	?,	0	?	?
Liver	0	0	0	0	0	?
Spleen	0	0	0	0	0	0
Brain	?	0	?	+	0	0
Muscle	0	0	0	0	0	0
Testicle	?	0	?	+	0	+

++++ = Marked absorption of antibodies.

+++ = Very distinct absorption of antibodies.

++ = Distinct evidence of absorption of antibodies.

+

= Slight evidence of absorption of antibodies.

? = Trace of absorption or questionable absorption of antibodies.

0 = No evidence of absorption of antibodies.

antigens the absorption by the spleen cells was overshadowed by the absorption with the cells corresponding to the antigen, or by absorption with other organ cells.

Testicle serum showed a slight but definite specific absorption when tested with testicle antigen. There was a suggestion of a specific absorption in brain antigen; here the specificity was, however, only relative. With the other four antigens there was no evidence of specificity.

Muscle antiserum did not show any evidence of specificity even when tested with muscle antigen, and there was only a very vague suggestion of specificity when tested with liver antigen. With the other antigens there was no suggestion of specificity. In fact we found throughout that muscle has very weak absorbent powers.

We therefore note that with the exception of muscle all the organs which we have tested tend to show a specific relationship between the antisera and the absorbent. The degree to which this relationship can be demonstrated, however, varies very markedly in the different organs.

We have previously noted that, at times, evidences may appear of a relationship between the tissue used as absorbent and its corresponding antigen, regardless of the antiserum used in the

experiment. Considering now this absorbent-antigen relationship, we find that in the kidney antiserum this relationship appears practically constantly; that is, in the four antigens and absorbents used with kidney serum. In spleen antiserum the relationship is also evident with kidney, liver and brain, but is not noted with testicle and muscle. In brain antiserum this is noted with spleen, kidney and testicle, but not with the other three tissues. However with even the first two the relationship is only relative and not clear and sharp. With liver antiserum the absorbent-antigen relationship appears quite definitely in the case of kidney but is only suggested in the cases of spleen and brain. With testicle antiserum this relationship appears quite definitely in the cases of kidney and brain, but not with the other organs. In muscle serum the relationship appears definitely with kidney and liver but with no other organs.

We see, therefore, that kidney, liver, spleen and brain all show this absorbent-antigen relationship quite markedly with most sera; kidney shows it possibly a trifle more definitely than the other three. However, testicle and muscle show it but very slightly, if at all. Furthermore, this relation between antigen and absorbent is noted most frequently when we use the antisera prepared against liver, kidney, spleen and brain and less frequently when we use muscle and testicle antisera.

If we compare the relative absorbent power of the various organs we note that liver and kidney both possess this power to a quite marked degree and in approximately an equal degree. Spleen and brain show only a fair degree of absorbent power and distinctly less than does either liver or kidney. Both testicle and muscle have a relatively low absorbent power.

If, now, we attempt to determine whether there exist any biological interrelationships between the various organs, as shown in our experiments, we must realize that here we can offer only tentative conclusions. It does appear that liver and kidney have a reciprocal relationship since both absorb very considerable quantities of immune substances from the other serum. Brain and testicle also seem to have a mutual relationship, since they both act upon the other serum. Even taking into con-

sideration the strong absorbent action of liver in general it does appear that liver bears some relationship to brain, but, on the other hand, there is no evidence that brain has any striking relationship to liver. Testicle seems to have no relationship whatsoever with spleen, muscle and liver, while muscle has no relationship to any of the other tissues. Testicle has, of course, a relationship to brain as mentioned above.

It seems possible, also, that liver, kidney, spleen and brain all have some sort of interrelations, but that muscle stands apart from these four, and testicle stands apart from all but brain. Maybe we can place the six tissues examined in our experiments in three groups; a first including liver, kidney, brain and spleen, shows varying degrees of interrelationship among the tissues included in it; a second group of which testicle is the example which bears relationship to only a very limited number of tissues, and a third group of which muscle is representative and which has relationship to practically no other tissues. We are here, of course, discussing not the general relationship which is common to all tissues of a given species but the relationships which seem to exist between individual tissues. It must be noted that we do not consider these latter relationships as proven to exist but simply point out here certain suggestive facts noted in our experiments; we hope at some later time to be able to examine further these facts.

DISCUSSION

In these experiments we have shown that there is a definite tissue specificity for each of the organs examined. It is true that this can not be shown in the case of all the tissues by each one of the various methods used; but at least by one or another of the methods it can be demonstrated in each case. Frequently it appears that the specificity is not absolute but is only relative; in other words, it is at times not clear and can only be made evident by comparison of results and by taking into consideration a number of different factors.

In some cases undoubtedly the specificity is weak while in others it is strong, and clearly evident; in some cases it may

possibly be quite marked but be disguised by the various relationships which seem to exist between the various organs. These relationships are not sharply defined and may be only apparent and not actual, so for the present we can only suggest the possibility that such relationships exist between the various tissues.

In addition, however, it does appear that the complexity of the biological¹ structure of an organ makes it difficult to demonstrate the tissue specificity. If we bear in mind that apparently kidney, liver, brain and spleen are all more or less related, have certain demonstrable immunological qualities in common, it is evident that specificity of any one of these organs can be made apparent only with difficulty.

We cannot from our experiments even venture an opinion as to wherein the specificity of any individual tissues resides, that is, whether it is a quality of one individual part of the tissue or of all parts of the tissue, or whether it is a quality of simple or complex chemical structures. It is, however, certain that the immunological structure of tissues is multigenerous, apparently able to stimulate the production of three types of immune bodies; first, the immune bodies common to the species, the substances showing species specificity; secondly, the immune bodies which are specific for the tissue, showing tissue specificity; and thirdly, the immune bodies which have a definite relationship with tissues other than the one under consideration. It appears that the relative activity of these three types of substances may vary in the different tissues; so that some may be strongly active as regards both the second and third types of substances, as are apparently liver and kidney; or like brain they may show marked activity as regards the second, and weak but varied activity as regards the third type, or like spleen they may be rather lacking in the second and also relatively weak in the third type. Testicle apparently shows definite but weak activity as regards the homologous organ specific substances, and but very little activity in connection with non-homologous organ specific substances. Muscle is apparently similar to testicle in both respects but the

¹ We have used the term biological throughout in order to express both immunological and chemical, including in the latter both analytical and stereochemical.

homologous specific type of substances seem to be even weaker. We are not in a position to suggest from our results anything regarding the relative quantities of the species specific substances in the various tissues.

It is possibly of interest to note that in the cases of the three tissues which showed specificity quite strongly, and therefore activity as regards the second type of substances, we were dealing with epithelial tissues, while in the case of the other three tissues, less active in this respect, we dealt in the case of spleen and muscle with tissues of mesenchymal origin, and in the case of testicle with a tissue of disputed origin. It is possible that this difference in activity corresponds with the greater differentiation of epithelial tissue as compared with connective tissue.

We cannot offer any explanation regarding the relationships which seem to exist between certain of the tissues; nor on the basis of our present experiments can we make more definite statements. It may possibly be that in some cases that relationship which may exist depends upon embryological origin of the tissues, or it may be dependent upon functional qualities of the tissues. These explanations, however, are offered only as suggestions.

We note further in these experiments a certain irregularity in the action of a given tissue as antigen, as antibody producing agent and as absorbent. These three functions do not run parallel in the individual tissues.

CONCLUSIONS

It has been possible by various methods to demonstrate tissue specificity in liver, kidney, spleen, brain, muscle and testicle. The methods of demonstrating this specificity and the degree of the specificity varies in the different tissues. In some cases the specificity is absolute, in others it is only relative.

The difficulty in demonstrating this specificity lies apparently in the extreme complexity of the biological composition of the tissues, and possibly in the interrelationship existing between various tissues.

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POLYVALENT ANTIBODY RESPONSE TO MULTIPLE ANTIGENS

F. M. HUNTOON AND S. H. CRAIG

From the Mulford Biological Laboratories

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The simultaneous production in a single individual of antibodies against each of a number of strains of bacteria or bacterial antigens is not only of theoretical interest but of practical value as well.

The employment of prophylactic immunization against disease and the use of immune sera to combat specific infection would be greatly facilitated if an adequate antibody production combined with a wide polyvalency could be assured.

The possible objections to such immunizations are entirely based on the theory that the simultaneous treatment with a number of antigens does not produce as great an antibody response to each antigen as can be obtained with single strains or antigens.

It is undoubtedly true in many instances that attempts at multiple immunizations fail to produce adequate antibody response. But it is not true in all instances, and this leads us to examine the factors which limit such polyvalent antibody production.

LIMITS OF POLYVALENT ANTIBODY PRODUCTION

Failure to produce an antibody response, either to single or to multiple antigens, can be attributed either (a) to a failure on the part of the immunity mechanism to respond, or (b) to factors influencing the general condition of the animal and indirectly the immunity mechanism. These are the phases of antibody production which we propose to discuss.

a. If a failure to respond on the part of the immunity mechanism is possible, it must be due to a limitation of the number of antigens to which such a response can be elicited or to a limitation of the degree of specific response to each antigen.

If this mechanism is non-specific, common to all antigens, but reacting to produce slightly different specific antibodies, then it is entirely conceivable that it could be so saturated with various antigens as to fail to respond to further stimulation. In such a case, there would be a limit to the number of antigens which could call forth a specific reaction.

The response of such a mechanism would have a limit and when stimulated to full capacity by a number of antigens, the specific response to each antigen would be lower than to complete stimulation by a single antigen.

If, on the other hand, such a mechanism is multiple, so that each antigen finds in the body a special field to which it has an affinity, and which on stimulation produces the specific antibody for that antigen, then the question of response to any antigen rests upon the existence of a suitable field. The volume of response, as far as the immune mechanism is concerned, should be the same, irrespective of whether one antigen or many are introduced.

b. It is recognized that, as a rule, an animal in poor general condition is not suitable for antibody production and it follows that the introduction of substances, which profoundly affect the general condition of the animal will militate against such response.

In immunization against bacteria or their products, where large amounts of bacterial emulsion or the unpurified poisonous products of their metabolism are introduced, the specific antigenic portion is necessarily accompanied by a large amount of non-specific substances, which may affect the animal's general condition.

Particularly in the use of multiple antigens is this true and this is one of the main causes of the failure to produce adequate polyvalent immunization.

It may then be stated that the possibilities of the production of polyvalent immunization depend:

1. On the adequacy of the immunity mechanism.
2. On the use of purified antigens, freed from toxic, non-specific factors influencing the general condition.

It is well known that the serum of animals and man normally contain antibodies against many of the pathogenic organisms (1); the reasons for this occurrence do not concern us at present, but the fact that so many different antibodies are present is evidence *per se* for the ability of the body to react to the stimulus of several antigens.

Castellani (2), one of the pioneers in the field of polyvalency, working with one, two, three and four antigens in various combinations has shown that there is no essential difference in the immunity response to any one antigen, irrespective of the number of antigens coincidentally injected.

The following curve tables are derived from an analysis of Castellani's results and each curve represents the average of the results obtained from all animals after similar treatments.

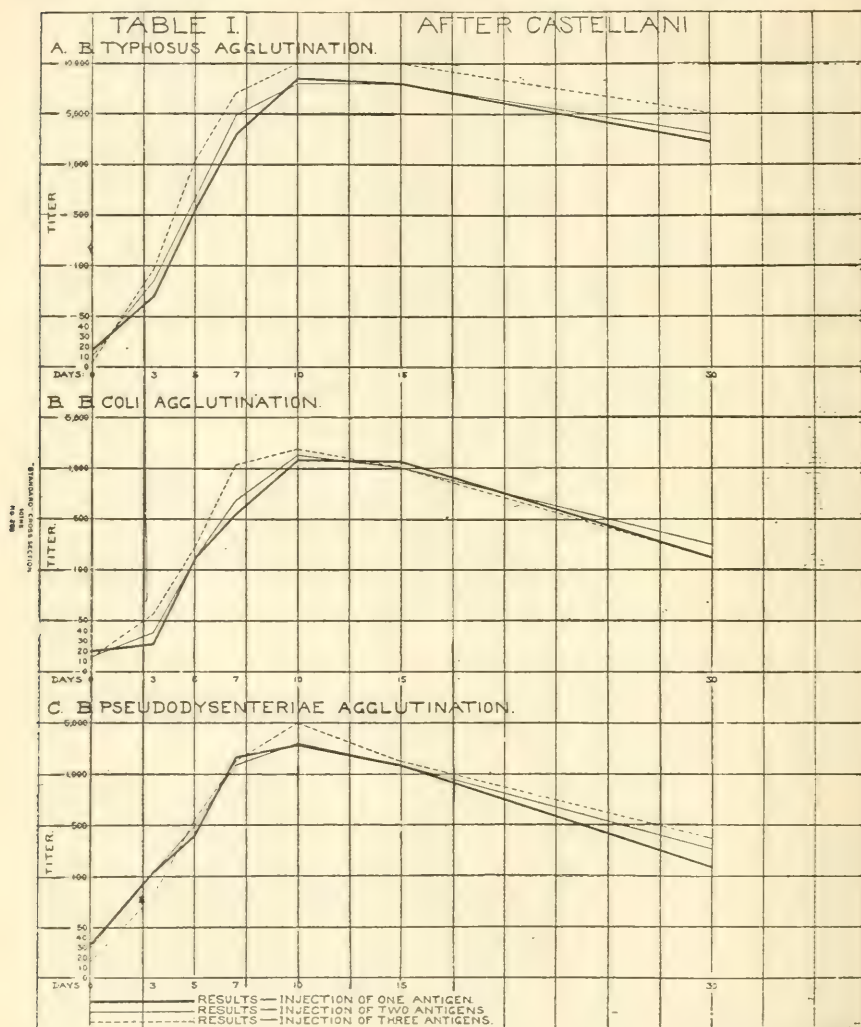
In table 1, A, B, C. The curves represent the agglutinating results of serum from rabbits injected with one, two and three coincident antigens; *B. typhosus*, *B. coli* and *B. pseudo-dysenteriae* in various combinations.

The curves are so arranged that there is a direct comparison of the agglutination of each antigen by sera produced by each method of immunization.

A glance at this table is sufficient to indicate the remarkable uniformity of the curves in each instance, although it is interesting to observe that in all of these sets, the curve of the triple antigen injection shows a slightly greater response than the others.

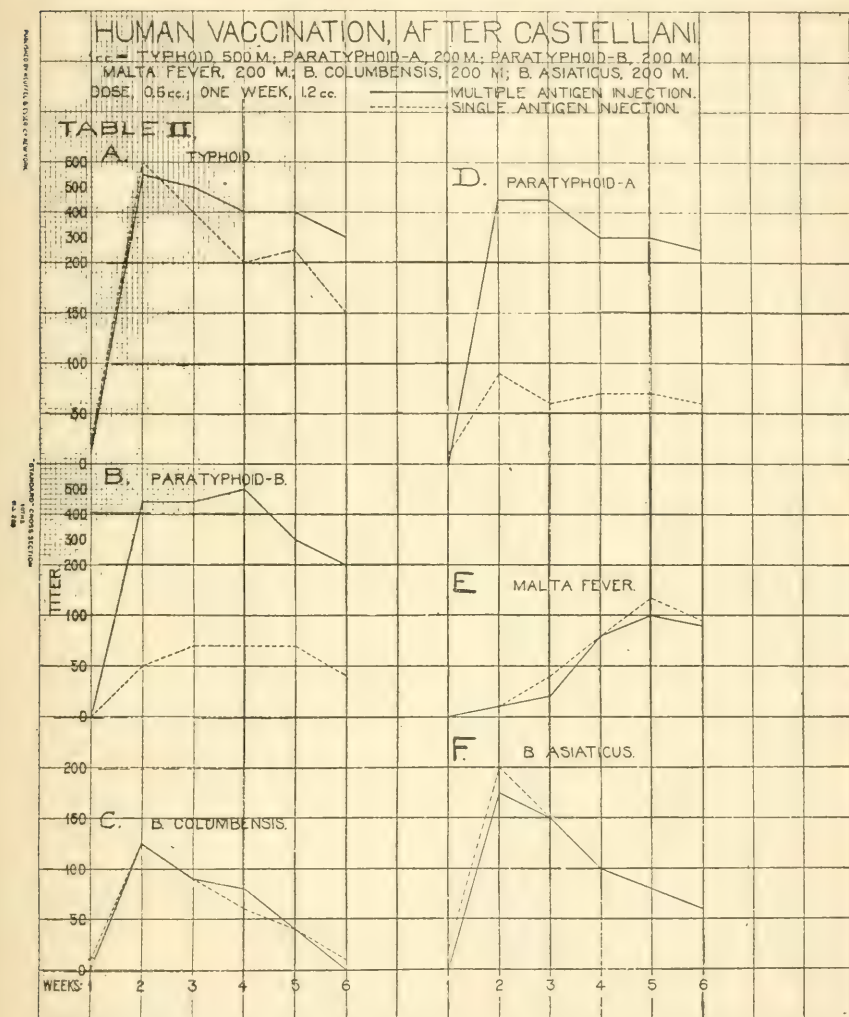
When this author attempted to add *Bact. prodigiosus* to the three antigens previously employed, most of his animals died. One, however, which survived five days, showed at this time an antibody production similar to that seen in the previous tests.

Castellani (3) in a further communication reports the use of multiple antigens in man controlled by the use of the homologous single antigens in other individuals. He employed from two



to six antigens given simultaneously and stated that such individuals in general produce agglutinins for each species of bacteria, in amount not much less than that produced by the single antigen controls (refer to table 2, A, B, C, D, E, F).

The results of multiple antigen injections presented in table 2 show that the response to each antigen of the six employed is equal to or greater than when the single antigens were employed.



Smith (4) reported a striking example of polyvalency in the production of ferments against bacterial antigens. Employing the Abderhalden technic he obtained the results shown in table 3.

Bull (5) produced in the same animal antitoxins of a fully competent strength against both tetanus toxin and that of the *Bact. perfringens*.

TABLE 3
(After Smith)

Protocol

Rabbits received repeated intravenous injections of single antigens at four day intervals.

Others received repeated injections of a mixture of all the antigens employed, at four day intervals.

Tests for presence of specific ferments; Abderhalden technique; rabbit serum

IMMUNIZING ANTIGEN	PERIOD AFTER LAST INJECTION	TEST ANTIGEN					
		Catarrh- alis	Influenza	Pneumo- coccus	Strepto- coccus	Staphy- lococcus aureus	Staphy- lococcus albus
	<i>weeks</i>						
Catarrhalis	1	++++	0	0	+	0	+
	2	++++	0	0	0	0	0
Influenza	1	0	++++	0	+	0	+
	2	0	++++	0	0	0	0
Pneumococcus	1	0	0	++++	0	0	0
	2	0	0	++++	0	0	0
Streptococcus	1	0	0	0	++++	0	0
	2	0	0	0	++++	0	0
Staphylococcus aureus	1	0	0	0	0	++++	0
	2	0	0	0	+	++++	0
Staphylococcus albus	1	0	0	0	0	0	++++
	2	0	0	0	0	0	++++
Mixture of above antigens	1	++++	++++	++++	++++	++++	++++
	2	++++	++++	++++	++++	++++	++++
Normal serum		0	0	0	0	0	0

O'Brien (6) produced simultaneous antitoxic antibodies against the toxins of tetanus, *Bact. perfringens*, *Vibrio septique* and *Bact. edemeticus*.

Reichel and Harkins (7) worked with thirteen strains of gram-negative colon-like bacilli (differentiated by fermentation reactions) which had been isolated from cases of "white scours" in calves. These observers found that as good response to each antigen could be obtained by injecting all of the strains into a single animal as where four strains or only one were employed.

Their results are given in table 4.

TABLE 4
(After Reichel and Harkins)

Protocol

Thirteen strains of Gram-negative bacilli from "white scours" calves (used for immunization of cattle).

Group A—4 strains.

Group B—4 strains.

Paracolon A, Paracolon B, *B. enteritidis*, *B. aerogenes*, and *B. metacolon*, one strain of each.

Monovalent serum: A separate animal employed for each strain.

Group serums: Animals immunized against 4 strains of group A. Animals immunized against 4 strains of group B.

Polyvalent serum: Animals immunized against all 13 strains.

Titer of various types of sera against the strains employed for immunization.

Sera drawn eight days after last injection.

	HOMOLOGOUS MONOVALENT SERUM	GROUP SERA	POLYVALENT SERUM (RECEIVED 13 STRAINS)
Group A—4 strains			
A.....	2,000	4,000	1,500
AI.....	1,000	1,000	1,500
AII.....	2,000	2,000	4,000
AIII.....	1,000	1,000	1,000
Group B—4 strains			
BII.....	200	200	400
BIII.....	32,000	2,000	8,000
BIV.....	16,000	4,000	8,000
BV.....	1,000	400	600
Bact. enteritidis.....	4,000		4,000
Bact. aerogenes.....	1,000		1,000
Metacolon.....	400		1,000
Paracolon A.....	800		800
Paracolon B.....	1,500		4,000

Davison (8), in an elaborate study of the effect of immunization with the *B. typhosus* and the *B. paratyphosus* A and B in both animals and man, makes the following statement: "When a mixed vaccine is used, the immunity obtained for each of its constituent bacilli is at least as good as, and often greater than, that obtained against any one employed alone in the same dosage."

TABLE 5

Comparison of polyvalent pneumococcus immunization with monovalent pneumococcus immunization

Protocol

Horses immunized for periods of three months to thirteen months with a mixture of the fixed types of pneumococci (first killed, and afterwards living organisms).

Injections tri-weekly by intravenous route

PNEUMOCOCCUS CULTURE	DOSE	POLYVALENT SERUM 6393		CONTROL MONOVALENT SERA		
		Serum	Mice	Serum	Mice	Control
	cc.	cc.		cc.		
Type I	0.01	0.2	S S	0.2	S S	Type I
	0.04	0.2	S S	0.2	S S	
	0.1	0.2	D(54) D(54)	0.2	D(92) D(92)	
Type II	0.001	0.2	S S	0.2	S S	Type II
	0.01	0.2	S S	0.2	S S	
	0.1	0.2	D(29) D(29)	0.2	D(38) D(16)	
Type III	0.001	0.2	S S	0.2	S S	Type III
	0.01	0.2	S S	0.2	S D(80)	
	0.1	0.2	S D(53)	0.2	D(15) D(15)	

S—Survived for more than ninety-six hours.

D—Death, with hours lived.

Virulence each strain—0.000,000,01 cc. killed mice in less than ninety-six hours.

As a matter of practice the present production of anti-meningococcic serum rests on a rather wide polyvalency, various producers employing from twelve to fifty strains to insure such results.

We desire to report here some of the results obtained by immunization of horses with multiple antigens in comparison with the results of the use of smaller numbers of antigens.

Three sets of animals (horses) were employed.

I. Pneumococcus immunization, with the fixed types 1, 2, and 3.

II. Pneumococcus and streptococcus immunization, with the fixed types of pneumococci and fifteen so-called key strains of streptococci.

III. Pneumococcus and streptococcus and *Bact. influenzae* immunization. The same as II and in addition ten strains of influenza bacilli.

The results of the polyvalent pneumococcus immunization are presented in table 5.

In the tables 6, A and B are shown the results obtained in the second series.

The results obtained with the third series are shown in tables 7, A, B, C, and D.

The results detailed above and those obtained by other workers in this field would appear to show:

1. That as yet no limit has been found as to the number of antigens which will elicit a simultaneous response.

2. That such responses approach in volume that obtained with single antigens.

Our experience with the multiple bacterial immunization of animals and with the coincident immunization of animals against toxins and bacterial antigens leads us to the conclusion that there is nothing in the immunity mechanism itself to preclude the possibility of a very wide polyvalent antibody production.

The undoubted fact that many animals under multiple antigen immunization at times fail to respond with as great a volume of production as occurs with single antigens may be explained by other factors such as a depreciation of the general physical condition due to the large amounts of bacterial protein injected.

If *all* animals failed to give adequate responses under multiple antigen immunization and the reduction of the volume of the response to each antigen bore a distinct relation to the number of antigens employed, it could then be inferred that the immunity mechanism itself was at fault and that true adequate polyvalency was impossible.

TABLE 6, A

Protocol

Horses which had been immunized against the fixed types of pneumococcus as shown in table 5 were in addition placed on increasing doses of streptococcus antigen containing equal portions of the fifteen so-called key strains.*

After six to eight weeks intensive immunization with both types of antigens, the animals were bled and the serum tested.

Protection tests against pneumococci

PNEUMOCOCCUS CULTURES	DOSE	POLYVALENT SERUM 5581		MONOVALENT SERA CONTROL (SEE TABLE 5)
		Serum	Mice	
	cc.	cc.		
Type I	0.01	0.2	S S	
	0.04	0.2	S D(40)	
	0.1	0.2	S D(19)	
Type II	0.001	0.2	S S	
	0.01	0.2	S S	
	0.1	0.2	S S	
Type III	0.001	0.2	S S	
	0.01	0.2	S S	
	0.1	0.2	D(27) D(19)	

Virulence each strain—0.000,000,01 cc. killed mice in less than ninety-six hours.

* Key strains of streptococci: These represent fifteen groups of streptococci obtained from human sources. Reported to the meeting of Society of American Bacteriologists, December, 1916.

TABLE 6, B

*Agglutination tests against streptococci**

TYPE STRAINS	POLYVALENT SERUM 5581 (SAME AS IN TABLE 8)				CONTROL STREPTOCOCCUS SERUM (RECEIVED NO PNEUMOCOCCUS)		
	C	100	200	400	100	200	400
2	—	4	4	4	4	4	4
3	—	4	4	4	4	4	4
4	—	4	4	2	4	4	4
5	—	4	4	4	×	×	×
6	—	4	4	4	4	4	4
7	—	4	4	4	4	4	2
8	—	3	2	—	—	—	—
9	—	4	4	4	4	3	—
10	—	4	4	4	4	3	2
14	—	4	3	—	4	3	—
1045	—	4	4	4	4	4	4
1046	—	4	4	4	—	—	—

TABLE 7, A

Polyvalent immunization with fixed types pneumococci, streptococci and influenza bacilli

Protocol

Horses at the height of their immunity to the fixed types of pneumococci were subjected to a coincident immunization with the streptococci as used in table 8, and in addition received 10 strains of influenza bacilli.

After six to eight weeks, bled; sera mixed and tested.

Protection tests against pneumococci

PNEUMOCOCCUS CULTURE	DOSE	POLYVALENT SERUM 82933		MONOVALENT SERA CONTROLS (SEE TABLE 5)
		Serum	Mice	
	cc.	cc.		
Type I	0.01	0.2	S S	
	0.04	0.2	S D(44)	
	0.1	0.2	D(20) D(18)	
Type II	0.001	0.2	S S	
	0.01	0.2	S S	
	0.1	0.2	D(18) D(17)	
Type III	0.001	0.2	S S	
	0.01	0.2	S S	
	0.1	0.2	S D(24)	

Virulence each strain—0.000,000,01 cc. killed mice in less than ninety-six hours.

TABLE 7, B

(For protocol see table 7, A)

*Agglutination tests against streptococci**

TYPE STRAINS	POLYVALENT SERUM 82933				CONTROL STREPTOCOCCUS SERUM		
	C	100	200	400	100	200	400
2	—	4	3	3	4	4	4
3	—	4	4	2	4	4	4
4	—	4	4	3	4	4	4
5	—	4	4	3	4	4	4
6	—	3	—	—	4	4	4
7	—	4	4	4	4	4	2
8	—	4	3	2	4	4	3
9	—	4	4	4	4	3	—
10	—	4	4	3	4	3	2
14	—	4	1	—	4	3	—
1045	—	3	2	1	4	4	4
1046	—	4	4	3	4	4	4

* Same antigens used for both tests.

TABLE 7, C
(For protocol see table 7, A)
Agglutination tests against influenza bacilli

TYPE STRAINS		POLYVALENT SERUM 82933					CONTROL INFLUENZA SERUM (10 STRAINS INFLUENZA ONLY)			
		C	80	160	320	640	80	160	320	640
Immunizing strains	143	—	4	4	2	2	4	4	3	3
	503	—	4	4	4	2	4	3	3	1
	509	—	4	4	4	4	4	4	3	3
	510	—	4	4	4	4	4	4	4	4
Non-immunizing strains	145	—	4	3	3	2	*	*	*	*
	498	—	4	4	3	3	4	4	2	2
	500	—	4	4	3	3	4	1	—	—

TABLE 7, D
(For protocol see table 7, A)
Protection tests against influenza bacilli

Emulsion: Four billion to 1.0 cc. (simultaneous injections of organisms and serum)

TYPE STRAIN	DOSE	POLYVALENT SERUM 82933	
		Serum	Mice
Immunizing strain 503	cc.	cc.	
	1.0	0.1	S S
	0.5	0.1	S S
		Virulence	
	0.5	0.0	D D
	0.1	0.0	D D
Non-immunizing strain 498	1.0	0.1	S S
	0.5	0.1	S S
		Virulence	
	0.5	0.0	D D
	0.1	0.0	D D

TABLE 7, E
(For protocol see table 7, A)
Complement fixation tests with influenza bacilli

ANTIGENS		POLYVALENT SERUM 82933			
		100	500	1000	2000
Immunizing.....	143	4	4	4	—
	503	4	4	4	2
	141	4	3	2	—
	143	4	4	4	4
	144	4	3	3	2
Non-immunizing	145	3	—	—	—
	499	3	2	2	—
	498	4	4	3	3
	500	4	4	4	4

The facts, however, point to an opposite view and the authors feel that proper preparation of such polyvalent antigen and the proper regulation of dosage will show that a polyvalency can be obtained sufficiently broad to cover the fields desired with an adequate concentration of antibody.

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A TEST-TUBE RACK FOR SEROLOGICAL WORK

OLIN DEIBERT

*From the Department of Bacteriology, College of Physicians and Surgeons,
Columbia University*¹

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A compact, convenient, and inexpensive test-tube rack, adaptable to many of the common bacteriological and serological procedures in which test tubes are involved, is herewith described, and a few of its advantages and possibilities indicated.

The simplest type of this rack, which was designed while the writer was working in the Department of Bacteriology at the University of Kansas, consists of a sheet of metal $5\frac{1}{2}$ by $4\frac{1}{2}$ inches bent at right angles along the greater dimension, $1\frac{1}{4}$ inches from the edge. The $1\frac{1}{4}$ inch portion of the sheet serves as a base, and the test tubes are held against the upright piece of metal by two rubber bands which are spaced an inch or more apart. In such a rack ten tubes of the common serological size (100 by 10 or 12 mm.) or a half dozen of the usual culture size may be handled as a unit, and the racks of tubes set together with the base of each rack resting on the base of the rack behind it in such a way that the successive rows of tubes are separated by scarcely more than the thickness of the metal sheet. For storing tubes in the smallest possible space, and yet maintaining ready accessibility, this style of rack has proved itself very useful. For carrying tubes in the pocket this simple rack is also convenient, since two or three racks full of tubes can be readily slipped into an ordinary coat pocket.

From the simpler rack was evolved the type herewith illustrated, combining many advantages not found in any other rack. The rack consists of a sheet metal base which rests flat upon

¹ Aided by a grant from the United States Interdepartmental Social Hygiene Board.

the table, and a strip of metal supported at right angles above the middle line of the base by two legs. The tubes are held against the upright back strip by rubber bands just as in the simple "L" rack, but the open space afforded by raising the back-strip away from the base by means of the two legs permits an unobstructed view of the lower ends of the test tubes.



FIG. 1

Although the exact details of construction may be greatly varied, the following description covers a model which is entirely satisfactory and at the same time readily set up in any shop. The base is a sheet of 28 gauge half-hard copper, $5\frac{1}{2}$ inches long and $1\frac{7}{8}$ inches wide. The two rear corners of this sheet are removed by cutting in a straight line from the middle point at each end

to points on the back edge of the strip about $1\frac{1}{4}$ inches from either end. The sheet is cut to a depth of $\frac{1}{4}$ inch at each end along a line drawn parallel to and $\frac{7}{8}$ inch from the front edge of the sheet. The tab thus formed is bent up at a right angle on a line $\frac{1}{4}$ inch from the end of the sheet. This $\frac{1}{4}$ inch upright tab serves as a point of attachment for the leg, which is a strip of



FIG. 2

the same material $\frac{1}{2}$ by $1\frac{3}{8}$ inches (or longer), bent double along its mid-line, and clamped to the base and back. The open space between the base and back is $1\frac{1}{4}$ inches. The upright back is made by bending a $5\frac{1}{2}$ by $1\frac{7}{8}$ inch piece of the copper sheet at right angles at each end along lines $\frac{1}{4}$ inch from the ends. These $\frac{1}{4}$ -inch portions serve to hold the rubber bands away from

the back so as to allow the tubes to be readily slipped into the rack, and at the same time do not prevent the rubber bands from holding the tubes firmly in place. The rubber bands should not be stretched too tightly, for their life will be greatly shortened thereby. This $\frac{1}{4}$ -inch portion extending forward at a right angle from the back also serves as a point of attachment for the leg, which is simply clamped on to the back and the base as it is being bent double. If the metal is clean at these points of contact, the parts may be firmly united by allowing a drop of solder to flow by capillarity into the space where the legs meet the back and base. The general construction of the rack is shown in the illustration, which, however, does not show that the base of the rack extends to the rear of the tubes almost an inch, so that the rack is prevented from falling over backward. The wide base ($1\frac{7}{8}$ inches) makes the rack quite stable and takes up very little space when the racks are grouped together.

When two or more racks are to be handled together, the base of the front rack is slipped over the base (and underneath the tubes) of the rack just behind, and the successive racks brought into contact. This grouping of the racks is especially convenient in handling large numbers of tubes in a minimum space. The grouping may be extended indefinitely, and a bunch of twenty or more racks picked up as a unit. A half-dozen or other convenient number of racks may be bound together as a unit by means of a rubber band, and a unit may be opened at any point to expose a desired rack for inspection just as a card index file discloses its individual cards. Inspection of tubes, as, for instance, in a series of agglutination tests, can be easily made, for the contents of all of the tubes in a given rack are entirely exposed to view, and it is unnecessary to remove any tube from the rack in order to give it a close examination, either by transmitted or reflected light. This feature combined with the ease of handling and the compactness makes the rack very serviceable for many kinds of work. Although it is possible for a tube to get out of place so as to change the order of a series set up in the rack, this does not occur in actual use. However, a diagonal grease-pencil mark drawn from the base of the

first tube to the top of the last will serve to indicate the proper position of the tubes in the rack and prevent any errors that might occur if a tube were shifted out of place.

The rubber bands are entirely satisfactory for all ordinary work, even when the racks are stored away for several months, and by using two or more bands the breaking of one of them does not endanger the contents of the rack. For carrying culture tubes to and from field work away from the laboratory the rack has proved itself very useful. If it were desired to use the rack for permanent storage of tubes of any sort, as for instance entomological specimens, the rubber bands could be substituted by cloth tape tied in place, and a permanent label could be painted upon the rack itself.

The space occupied by these racks is about one-fourth that required by the ordinary metal racks such as are commonly employed in Wassermann tests, handling the same number of tubes in both cases.

It is a pleasure to express my indebtedness to Dr. Oscar Teague for the encouragement and valuable suggestions which he has given.

IMMUNOLOGICAL EXPERIMENTS WITH CATALASE¹

THEO. C. BURNETT AND CARL L. A. SCHMIDT

From the Department of Physiology and the Department of Biochemistry and Pharmacology of the University of California, Berkeley

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A property possessed by ferments which distinguishes them as a class from catalysers which are not products of the living cell, is the ability, when repeatedly injected into animals, to give rise to substances which inhibit the action of the ferment (1). The inhibitory substances are termed antienzymes. Although the chemical makeup of enzymes is not known, it appears probable that they are protein in nature and it is to this that their ability to give rise to immune bodies can be attributed. Immune bodies cannot however be produced for all proteins. Thus gelatin (2), the protamines (3), and the lower proteoses (4) are not antigenic. It is therefore conceivable, if ferments are protein in nature, that certain ferments may exist for which antibodies cannot be produced.

While antibodies inhibitory to the common enzymes are known, investigators have noted in several instances, that repeated injection of ferment does not lead to the appearance of anti-ferment in the blood stream of the injected animal. Thus De Waele and Vandeveldt (5) were unable to immunize animals against catalase, a ferment found widely distributed in both plant and animal cells and which possesses the ability of accelerating the decomposition of H_2O_2 according to the reaction $2\text{H}_2\text{O}_2 = 2\text{H}_2\text{O} + \text{O}_2$. Similarly von Fürth and Jerusalem (6) report that the serum of a rabbit which had been given two injections of a tyrosinase solution did not inhibit the action of this enzyme in oxidizing tyrosin. The latter investigation is seriously questioned by the more careful work of Gessard (7)

¹ Aided by a grant from the Research Fund of the University of California.

who prepared both antityrosinase and antilaccase sera and showed that the inhibitory action was specific.

The production of inhibitory antibodies by immunization must of course not be confused with the naturally occurring ferment inhibitory substances found in animal tissues. It is a matter of doubt as to whether the factors concerned in the anti-tryptic action of normal blood sera and in immune sera are identical. With reference to catalase, Battelli and Stern (8) state that they found in the extract of tissues a substance of ferment nature which they term anticatalase and which possesses the property, in the presence of oxygen, of destroying catalase. They also believe that a substance, philocatalase, which possesses the power of destroying anticatalase, occurs in normal tissues. Czapek (9) finds that the antioxydases found in plants possess a marked species specificity. Although Battelli and Stern (10) did not carry out immunological experiments with catalase they found on injecting catalase in large amounts that it is non-toxic and that it rapidly disappears from the blood stream and tissues, due, as they believe, to its destruction by anticatalase.

From the evidence available we are led to the conclusion that oxydases are not antigenic while the peroxidasess (a group including tyrosinase and laccase) whose function appears to consist in facilitating the transfer of oxygen from peroxides to substrate, are not only antigenic but also possess a marked specificity. Our interest has been directed towards carrying on further work to ascertain whether repeated injection of catalase into rabbits leads to the appearance of an antibody which will inhibit the decomposition of H_2O_2 by catalase and while our results do not answer this question in the affirmative they nevertheless throw some interesting light on the result of repeatedly injecting this enzyme into animals.

Catalase was prepared (11) from both calf and ox liver by reducing the tissue to pulp in a meat grinder and then shaking for several hours with an equal volume of H_2O . The mixture was strained through linen and the residue was reextracted with H_2O and combined with the first portion. The combined extract was slowly poured into about 5 volumes of acetone which resulted

in the formation of a voluminous precipitate. This was filtered off on hardened paper and the acetone was evaporated in a current of air. The residue was extracted with several volumes of water, precipitated by pouring into acetone as before, washed with acetone and dried *in vacuo* over H_2SO_4 . The preparation thus obtained was very active in the liberation of oxygen from neutral H_2O_2 . Like most ferment preparations catalase prepared by this method is grossly contaminated with tissue proteins which however should not interfere with the production of specific antibodies.

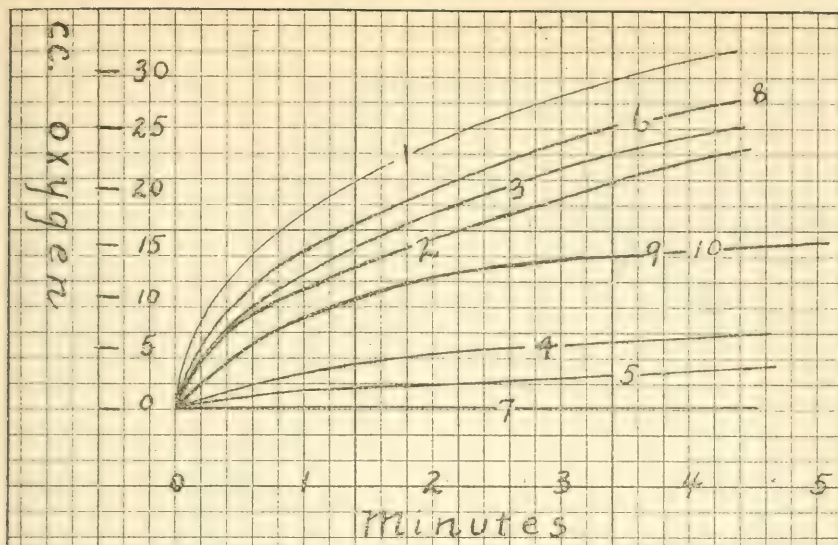
For purposes of injection and in testing its activity, catalase was triturated with salt solution, a small amount of insoluble protein was removed by centrifuging and the solution was filtered. Four rabbits were given alternate intravenous and intraperitoneal injections of catalase in 50 mgm. doses. Two animals received a total of 250 mgm. each and the other two 800 mgm. and 1 gram respectively. The clear unhemolyzed sera of these animals were used in testing for antibodies to catalase.

To measure the activity of the ferment in accelerating the decomposition of H_2O_2 we used an apparatus somewhat similar to that described by Baas Beeking and Hampton (12) by means of which not only the total volume of oxygen given off but also the reaction time is obtained and automatically recorded by means of a kymograph. The catalase solution was measured into a test tube and to this a separatory funnel containing a given volume of carefully neutralized 50 per cent H_2O_2 was connected by means of a rubber stopper. Through the other hole of the stopper a glass Y-tube was inserted, one end of which was connected by means of rubber tubing to a volume recorder: the other end was similarly connected to the mouth of the separatory funnel. In this way H_2O_2 can be added to the catalase solution without causing a volume change. The entire apparatus was placed in an air thermostat and the catalase- H_2O_2 mixture was shaken by means of a slow speed motor.

Into a number of test tubes varying amounts of sera from a normal and from the injected animals were pipetted and to each a constant volume of catalase solution was added. The mixtures

were brought to constant volume by addition of salt solution and then incubated for one-half hour. This resulted in the appearance of a flocculent precipitate in each of the test tubes containing sera from the animals injected with catalase, the amounts varying with the amount of serum added. This phenomenon was not unexpected since the liver proteins are known to be antigenic (13). The precipitate was centrifuged and, on testing the supernatant fluid, a decided decrease in catalase content was noted, the amount varying with the quantity of precipitate. By adding a sufficient quantity of serum the volume of oxygen liberated from the H_2O_2 was reduced to zero. No precipitate was evident on adding normal rabbit serum to catalase. The results of these experiments are shown graphically in figure 1. If, instead of centrifuging the precipitate and testing the supernatant fluid for catalase, the mixture was shaken so as to secure a uniform distribution of precipitate, no reduction in the volume of oxygen liberated was found and the reaction time was the same as was noted for catalase (experiment E). The experiments were repeated with antigen obtained from guinea-pig liver, the dilution being so adjusted that its activity corresponded with the other catalase preparations. Similar results were obtained although the antisera were not quantitatively as effective as when the homologous antigen was used. Catalase was added to a precipitin system containing human and rabbit vs. human serum, the mixture was incubated and the precipitate was removed by centrifuging. On testing the supernatant fluid for catalase no decrease in activity was noted. Catalase was not carried down by the globulin precipitate produced by adding normal rabbit serum to catalase solution, diluting and passing in CO_2 . As stated before only neutral H_2O_2 was used in these experiments (the commercial preparations are very acid) and in certain of the tests, neutral phosphate buffer mixture was added to eliminate the possible factor of acidity.

It does not appear probable that catalase is mechanically removed from solution (as pepsin is by calcium phosphate precipitate) by the precipitate formed on addition of serum from the injected animals, since no catalase was removed from solution



CURVES SHOWING THE AMOUNT OF OXYGEN GIVEN OFF IN THE CATALYTIC DECOMPOSITION OF H_2O_2 WITH TIME

Note the marked decrease in the amount of oxygen as a result of the precipitation of ferment by catalase.

Experiment A. Curve 1. Calf liver catalase.

Curve 2. 0.5 cc. serum 13 added to an equal volume calf liver catalase. Precipitate removed by centrifuging.

Curve 7. 0.2 cc. serum 3B added to 0.5 cc. calf liver catalase. Precipitate removed by centrifuging.

Experiment B. Curve 3. Guinea-pig liver catalase.

Curve 4. 0.2 cc. serum 3B added to 0.5 cc. guinea-pig liver catalase. Precipitate removed by centrifuging.

Experiment C. Curve 5. 0.5 cc. serum 3B added to 0.5 cc. guinea-pig liver catalase. Precipitate removed by centrifuging.

Curve 6. Same as above. Precipitate suspended.

Curve 8. Guinea pig liver catalase.

Experiment D. Curve 9. Calf catalase.

Curve 10. Addition of human and rabbit vs. human precipitin system to calf catalase.

Note: 6 cc. 50 per cent H_2O_2 used in all experiments. The initial concentration of catalase was adjusted by dilution to a constant for each experiment.

Experiment E. Varying amounts of "immune serum" were added to a constant amount of catalase and the volumes were brought to a constant by addition of salt solution. The precipitate was removed by centrifuging and the catalytic activity of the supernatant fluid was determined by noting the volume of oxygen which was liberated from 8 cc. of H_2O_2 in five minutes.

Serum no. 3A added cc.	Volume of oxygen liberated cc.	Serum no. 1 added	Volume of oxygen liberated cc.
None	34.7	None	26.0
0.1	15.4	0.4	2.5
0.2	5.6	0.4 (precipitate shaken)	25.0
0.3	3.4		
0.5	2.1		
0.2 (normal serum)	34.3		

by the precipitate in the human system. On the other hand the evidence does not warrant the assumption that a specific precipitin for catalase is present in the sera of the injected animals. More probable is the hypothesis that catalase is combined with the liver proteins present in our preparation and that the complex is the antigen leading to the formation of a precipitate. The phenomenon appears to be somewhat analogous to the precipitation of diphtheria and tetanus antitoxin when added to the serum of a rabbit immunized to horse serum (14).

Our experiments do not indicate the presence, in the sera of the injected animals, of an antibody which can inhibit the decomposition of H_2O_2 by catalase. A number of explanations for this phenomenon might be suggested although direct proof is in all instances lacking. However, it is an established fact that immunization with certain toxic proteins does not lead to the appearance of inhibitory antibodies although specific agglutinins may be produced. It is possible that the toxin is combined with other proteins and that the presence of the latter in the antigen complex gives rise to the production of agglutinins. An example is found among that indefinite group of substances known as endotoxins. Animals immunized against bacterial proteins with which these toxic substances are associated develop in their sera bactericidal and agglutinating antibodies but it is difficult or impossible to obtain an antitoxin for the endotoxin (15). Although the catalase is carried down by the precipitate produced on adding serum from the injected animal to catalase solution, the catalase nevertheless remains free to act on H_2O_2 . In this respect the phenomenon is analogous to that noted by Gay and Chickering (16). They found that the washed precipitate produced by adding homologous antiserum to the aqueous extract of pneumococci protects as well as the whole serum. It is not probable that catalase is slowly dissociated from the precipitate when the latter is added to H_2O_2 since the reaction time curves are identical with those of catalase. The other alternative is either instantaneous dissociation or that catalase although bound to the precipitate complex is free to act by virtue of its terminal position in that complex. The latter idea is not

new. An analogy is found in the experiments described by Gay and Robertson (17) and by Schmidt (18) who found that rabbits immunized to compound proteins yield antibodies not only for the complex but for the individual components as well. Evidently the components of the compound are still able to act individually and thus stimulate the production of antibody.

SUMMARY

The sera from rabbits which had received repeated injections of catalase prepared from calf liver, when mixed with the homologous antigen, gave a precipitate which carried with it catalase from the solution, in amounts dependent on the quantities of sera used. There was no evidence that a true inhibitory antibody was present in the sera of the injected animals, since the precipitate was as active as the antigen in the liberation of oxygen from neutral H_2O_2 . The theoretical aspects of the results are discussed.

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THE VALUE OF TISSUE EXTRACTS OF VIRUS PIGS IN THE PRODUCTION OF ANTI-HOG CHOLERA SERUM

THOMAS P. HASLAM¹

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I. INTRODUCTION

The experiments presented in this article were planned to ascertain whether expressed or extracted juices from the tissues of "virus pigs" could be used as a means of hyperimmunizing hogs in preparing anti-hog cholera serum by the Dorset-Niles method. Uhlenhuth (1) seems to have considered that the saline extract of the blood containing organs was about equal to the blood itself as a means of hyperimmunization. However his methods of hyperimmunization and of testing the potency of the serum vary so greatly from American methods that it is difficult to judge what might be expected under the conditions prevailing here. Our experiment was limited to muscle tissue because the juices recovered from the visceral organs contained many more bacteria than the juices recovered from the musculature.

II. PREPARATION OF MUSCLE VIRUS

The pigs killed for virus in the routine production of anti-hog cholera serum were suspended head downward immediately after being killed. The skin covering the hams was washed with anti-septic and shaved. An incision was made, the skin was grasped with powerful forceps, and pulled off from the carcass. The muscle tissue was removed with sterile instruments and ground in a sterile "Enterprise" sausage grinder. The muscle virus

¹This work was done while the author was with the Kansas Agricultural Experiment Station, Manhattan, Kansas.

for hyperimmunization experiments was obtained from the ground meat in three different ways.

Process 1. The ground meat was frozen and subsequently thawed in such a manner that the juices flowed away drop by drop as the thawing progressed, yielding about 100 cc. of clear, undiluted, muscle virus per pound of meat.

Process 2. The ground meat was mixed with an equal volume of physiological salt solution, and treated as in process 1, yielding about 400 cc. of clear, reddish extract per pound of meat.

Process 3. The ground meat was mixed with an equal amount of physiological salt solution the mixture chilled, and after standing twenty-four hours pressed through sterile canvas by means of a small screw press, yielding about 400 cc. of a turbid, light colored, milky extract per pound of meat. This was strained through several thicknesses of gauze before injection.

III. METHODS OF HYPERIMMUNIZATION

Muscle viruses prepared by the three processes described, were administered intraperitoneally, intravenously and subcutaneously. The intraperitoneal method was found undesirable because of faulty absorption. The intravenous method was likewise undesirable in our experience.

In administering these muscle virus extracts intravenously it was necessary to give them very slowly because muscle extracts are rich in lymph and if injected rapidly produce an intravascular coagulation of blood, and death of the hyperimmune animals. Centrifuging the extracts in order to free them from small bits of tissue which, injected intravenously, might act as emboli, somewhat reduced their injurious properties. Better technique in the slow intravenous injection would doubtless largely reduce the loss from this source. The experiments of Dold (2), which were unknown to the writer till later on in the experiments, have demonstrated that intravascular coagulation does not take place when the lymph containing fluid is injected gradually. The Woodyatt pump would probably be ideal for this purpose as the rate of injection could be exactly controlled. It is probable that a mixture of blood virus and muscle virus could be more readily administered than muscle virus alone, since Dold (2) has shown that normal

blood is capable of neutralizing those properties of the lymph which produce the intravascular coagulation of the blood on intravenous injection. A few hogs immunized with a mixture of muscle virus and blood virus in our own experiments withstood the injection nicely, but on this point our experiments are not sufficiently complete to warrant presentation. The logical procedure seemed to be to test the hyperimmunizing value of the muscle extracts, before proceeding with mixtures.

In our work the muscle virus was usually administered subcutaneously. The amount of muscle virus given was 10 cc. per pound weight of the animal, since Dorset and Niles demonstrated that this amount of blood virus given subcutaneously would produce a good serum. The number of abscesses which developed following the subcutaneous injection of muscle virus seemed to be no higher than that usually observed following the injection of blood virus subcutaneously.

IV. TESTS ON SERUM PREPARED FROM MUSCLE VIRUS

The plan of the experiment called for testing the potency of the serum from hogs hyperimmunized with muscle virus. The potency test consisted of inoculating 40 to 50-pound test pigs simultaneously with 2 cc. of ordinary defibrinated blood virus and a definite amount of the serum to be tested. In every instance a serum control test on regularly prepared serum was made, and also a test of the virus. This was in an attempt so to control conditions that the only variable present was the serum to be tested. Only those experiments will be recorded in which serum was produced from both muscle virus and blood virus of the same lot of pigs. A few experiments in which the blood virus failed to produce a potent serum are omitted, but all experiments are reported in which potent serum prepared from blood virus and serum prepared from the muscle virus of the same lot of pigs were simultaneously tested. Complete temperature and symptom records were kept, but only summaries will be given.

Six complete experiments were carried out. Each experiment consisted of three groups of pigs. One group received serum from hogs hyperimmunized with muscle virus. The second

TABLE 1
Potency tests of serum prepared from muscle virus

KIND OF SERUM USED	METHOD OF HYPERIMMUNIZING TO PRODUCE THE SERUM	POTENCY TEST			RESULTS
		Num- ber of pigs	Serum	Virus	
Experiment 1					
Muscle virus serum	Process II, muscle virus intravenously, 6 cc. per pound	4	20	2	Remained well
Check serum	Blood virus intravenous- ly, 6 cc. per pound	2	20	2	Remained well
No serum		6	None	2	All sickened 7th day
Experiment 2					
Muscle virus serum	Process II, muscle virus intravenously, 10 cc. per pound	2	20	2	Remained well
Check serum	Blood virus intravenous- ly, 6 cc. per pound	2	20	2	Remained well
No serum		2	None	2	Sickened 7th to 8th days
Experiment 3					
Muscle virus serum	Process III, muscle virus subcutaneously, 5 cc. per pound and 12 days later subcutaneously, 8 cc. per pound	3	15	2	Remained well
Check serum	Blood virus, two subcu- taneous treatments*	3	15	2	Remained well
No serum		2	None	2	Sickened 5th to 7th days
Experiment 4					
Muscle virus serum	Process I, muscle virus subcutaneously, 10 cc. per pound, one bleeding only	2	15	2	Remained well
		4	20	2	Remained well
		2	25	2	Remained well
Check serum	Blood virus, 10 cc. per pound subcutaneously, one bleeding only	2	15	2	Remained well
		2	20	2	Remained well
		2	25	2	Remained well
No serum		6	None	2	All sickened 5th to 8th days

* Six cc. was given one day and 4 cc. the next.

TABLE 1—Continued

KIND OF SERUM USED	METHOD OF HYPERIMMUNIZING TO PRODUCE THE SERUM	POTENCY TEST			RESULTS
		Num- ber of pigs	Serum	Virus	
Experiment 5					
Muscle virus serum	Process I, muscle virus subcutaneously, 6 cc. per pound	2	15	2	Remained well
		2	20	2	Remained well
		2	25	2	Remained well
Check serum	Blood virus intravenous- ly, 6 cc. per pound	2	15	2	Remained well
		2	20	2	Remained well
		2	25	2	Remained well
No serum		6	None	2	All sickened 5th to 7th days
Experiment 6					
Muscle virus serum	Process I, muscle virus subcutaneously, 6 cc. per pound and 13 days later 8 cc. per pound	3	20	2	Remained well
Check serum	Blood virus, 6 cc. per pound intravenously	3	20	2	Remained well
No serum		2	None	2	Sickened 6th day
Field test using heavier pigs					
Muscle virus serum	Process I, muscle virus as in experiment 6	2	35	2	Died
		2	70	2	Remained well
Check serum	Blood virus as in experi- ment 6	2	35	2	Remained well
		2	70	2	Remained well

group, referred to as serum controls, received serum prepared by hyperimmunizing hogs with 6 cc. per pound of blood virus from the pigs from which the muscle virus was prepared. The third group referred to as virus checks did not receive any serum, but received 2 cc. of virus only. A summary of the results obtained in the six experiments is presented in table 1.

Experiment 7 differs from all other tests in that it was the mixed serum from a considerable number of hyperimmunes, hyperimmunized at different times, and in different ways. On

TABLE 2
Tests on a mixture of all lots of muscle virus serum

KIND OF SERUM USED	METHOD OF HYPERIMMUNIZING TO PRODUCE SERUM	TEST			RESULT
		Num- ber of pigs	Serum	Virus	
Experiment 7					
Muscle virus serum mix- ture	Muscle virus by different processes and methods	2	cc. 15	2	Remained well
		2	20	2	Remained well
Check serum	Made by Bureau of Ani- mal Industry at Ames, Iowa	1	15	2	Remained well
		2	20	2	Remained well
No serum		2	None	2	Sickened 6th to 9th days
Second test					
Muscle virus serum mix- ture	Muscle virus by different processes and methods	2	15	2	1 sickened 11th day and died 15th day; post mortem pneu- monia. 1 re- mained well
		3	20	2	Remained well
		3	25	2	Remained well
Check serum	6 cc. blood virus per pound intravenously	2	5	2	Sickened 6th to 8th days
		2	10	2	1 sickened 6th to 10th days but recovered 1 sickened 6th day, died 14th day
		2	15	2	Remained well
		2	20	2	Remained well
No serum		2	25	2	Remained well
		4	None	2	Sickened, killed 8th day
Third test					
Muscle virus serum	Muscle virus by different methods	4	15	2	Remained well
		4	20	2	Remained well
		4	25	2	Remained well
No serum		4	None	2	Sickened, killed for virus 8th day

account of the shortage of test pigs, each lot of serum could not be tested separately; therefore, aliquot parts of each individual lot of serum were taken and mixed together, this making a sample representing 200,000 cc. of serum prepared from muscle virus. This serum was checked against serum furnished by the United States Bureau of Animal Industry, and retested later against another standard serum. After the writer had discontinued these experiments this 200,000 cc. of serum was again tested by Dr. C. W. Hobbs, and the writer is indebted to Dr. Hobbs for the data recorded in table 2 as the third test on the muscle virus serum mixture.

V. DISCUSSION

From the record in table 1, it is seen that six lots of serum made from muscle virus were tested, and for each lot a test was run on serum made from the ordinary or blood virus of the same virus pigs. Fifteen or 20 cc. of the serum made by hyperimmunizing with muscle virus was in every instance able to protect 40 or 50-pound test pigs against 2 cc. of ordinary, phenolated, defibrinated blood virus. This was also true of the control blood virus serum. The vaccinated pigs were kept under examination for fifteen days. The virus checks in every instance promptly developed cholera and showed marked sickness, "broke" in from five to eight days. In one instance, experiment 6, a further test was disappointing. Some heavier shoats were vaccinated and turned out in a feed lot. The serum made from the muscle virus failed to protect two of the shoats which received only 35 cc. of serum, and in this instance showed less favorable results than the serum prepared from the blood virus. The muscle virus from which this serum was prepared had been stored in a frozen condition for several weeks prior to the hyperimmunizing of the hogs, and this may have influenced the results.

The data presented in table 2 is of even more importance than the data in table 1, for it records the result of tests made on a 200,000 cc. mixture of all serum prepared from muscle virus. This serum mixture was tested at three different times. A total of twenty-four pigs received 15 to 25 cc. of muscle virus serum and

2 cc. of ordinary blood virus. All remained well except one which sickened the eleventh day, and died the fifteenth day and showed pneumonia on post mortem. Nine pigs received 15 to 25 cc. of check serum and 2 cc. of blood virus, and remained well, but two pigs that received 10 cc. of check serum and 2 cc. of blood virus sickened.

CONCLUSIONS

1. The experiments here recorded show that serum of considerable potency may be prepared from virus derived from the muscle tissue of virus pigs.

2. The methods of testing the potency of serum are so crude that it is difficult to compare the potency of two sera without the use of a large number of test animals. The original plan of the experiment called for a test according to the principles previously established (3) by the author, but the necessary number of pigs was not at my disposal so the test had to be abandoned.

3. The toxicity of muscle virus on intravenous injection is the principal obstacle in the way of practical application of muscle virus in the manufacture of anti-hog cholera serum, but this may be combated by slow injection, or preferably by mixing with blood virus.

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OBSERVATIONS ON THE AGGLUTINATIVE AND HEMOLYTIC ACTION OF CALF SERUM ON SHEEP CELLS¹

FRANK MALTANER AND ELIZABETH JOHNSTON

From the Division of Laboratories and Research, New York State Department of Health, Albany. Augustus Wadsworth, M.D., Director.

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The practical importance of the effect of hemoagglutination upon the accurate estimation of complement fixation in complement fixation tests has been brought to our attention with special emphasis in a study of the significance of the complement fixation reaction in calves infected with tubercle bacilli.

During this experimental study the sera of four normal calves were tested for their agglutinative and lytic action upon sheep cells. Washed sheep cells suspended in saline were used both alone and in the presence of complement (fresh guinea-pig serum), and also a similar suspension sensitized with anti-sheep cell amboceptor. The active serum of these four calves strongly agglutinated the sheep cells in all of these tests. The serum of one of the calves, in addition, strongly hemolysed the sheep red cells while only slight or no hemolysis was caused by the sera of the other three calves. When the sera were inactivated at 56°C. for thirty minutes in the water-bath considerably less agglutination and hemolysis were produced. Attempts to prevent this agglutination and hemolysis by absorption of the calf serum with sheep cells were unsuccessful except occasionally when a large excess of washed, packed sheep cells was used.

The literature contains records of innumerable studies which indicate the agglutinative character of normal bovine sera for many different bacteria and for the red blood cells of practically all domestic and laboratory animals. No detailed study of the

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agglutinative action of bovine sera upon sheep cells, however, has been reported. Bordet and Gay (1) have found that bovine serum also possessed a strong agglutinating property, that is, strong agglutination and sometimes lysis of red blood cells or bacteria are observed when inactive bovine serum is mixed with the active sera of several other animals, none of which in the absence of bovine serum caused any marked agglutination or lysis of the bacteria or the red blood cells used. Bordet (1) was able to reduce the agglutinating action of bovine sera for guinea-pig red blood cells by absorption of the serum with sensitized and alexinized guinea-pig cells.

In some experiments undertaken by Miss Hoppe in this laboratory to determine the effect of calf serum upon the phagocytic activity of calf leucocytes, the serum leucocyte mixtures were found to clot. This clotting was avoided by washing the suspensions of calf leucocytes free from blood platelets. The results of Miss Hoppe's experiments indicated that during the clotting of calf serum the cytozyme was first used up and that some of all of the other elements remained in the serum after clotting. The subsequent addition to the serum of cytozyme in the form of blood platelets produced a second clot. These observations taken together with recently published studies of Bordet (2) upon the mechanism of blood coagulation suggested to us that the agglutination of the sheep cells might be due to the blood platelets present in the suspension, and that the phenomenon was caused by a secondary clot-formation in the calf serum induced by these platelets. According to Bordet (2), the clotting of blood is produced by a combination of elements all of which must be present before clotting will take place. These elements are proserozyme, which in the presence of the soluble calcium salts of the blood and contact with a foreign body, forms serozyme, and is derived from the plasma constituents; fibrinogen, which also is contained in the plasma; and cytozyme which is furnished by the cellular elements of the blood, principally by the platelets, and which reacts with serozyme to form thrombine. The thrombine is the active principle which converts, in the clotting phenomenon, the fibrinogen into its insoluble form, fibrin.

As far as we have been able to ascertain, the literature contains no reference which associates the agglutinating properties of normal serum with clot-formation, although several workers have maintained that the cells or the bacteria play a more or less passive part in this phenomenon. Paltauf (3) and Kraus and Seng (4) associated agglutination by immune serum with the formation of specific precipitates by reaction of the serum with dissolved protein within and around the cells (bacteria, etc.), which they believed enmeshed and carried down the suspended cells. Arkwright (5) had a similar view and extended it to include acid agglutination. Priestly (6) associated agglutination with serum protein probably the globulin of immune serum which he believed to be altered in its electrical or adsorptive properties by reaction with the protein of the suspended cells thus rendering them amenable to precipitation by electrolytes. Dean (7) demonstrated that the addition of euglobulin from guinea pig serum or of fresh serum to anti-typhoid immune serum increased the agglutinating titer to a marked degree.

In order to determine whether the agglutination of sheep cells by calf serum was the result of secondary clot formation the following experiments were carried out. Calf serum was treated with cytozyme in the form of blood platelets obtained from sheep's blood. This treatment caused a secondary clotting of the serum. After removal of this secondary clot the serum no longer agglutinated sheep cells, either alone or in the presence of complement or of sensitized sheep cells. In addition the hemolytic properties of the calf serum had completely disappeared. On account of the anti-clotting effect of sodium citrate and oxalate, solutions of these salts were added to mixtures of serum and sheep cells in the presence and absence of complement and to sensitized sheep cells. By this procedure agglutination was prevented and hemolysis partially inhibited. (In carrying out this test it is necessary to add the citrate solution to the serum cell mixtures and not make up the washed cell suspension in citrated salt solution on account of the agglutinating action of citrate solution for sheep cells in the absence of serum.) The attempt was also made to remove the cytozyme from the sheep's cell suspension

in order to determine whether the agglutinative action of the calf serum would be eliminated or diminished by this procedure. The blood platelets and leucocytes were removed as carefully as possible from the sheep cells. However, the agglutination by the calf serum of the suspensions prepared from these platelets and leucocyte free cells was only slightly lessened by this procedure and the lysis was apparently not affected. The failure to reduce the agglutination and lysis to any marked degree was undoubtedly due to the limitations of the technic used in removing the cytozyme containing elements from the sheep cell suspension.

The detailed technic together with the experimental results and protocols are as follows:

EXPERIMENTAL DETAILS AND PROTOCOLS

The reagents

Calf serum: Calves were bled from the jugular vein, with sterile precautions. The blood was collected in shallow layers 2 to 3 cm. deep in flat-bottom flasks and allowed to stand in a cool place over night. The clear serum was removed from the clot and centrifugalized strongly to remove all of the suspended material.

Sheep cell suspension: Defibrinated sheep's blood was washed five times with 0.85 per cent salt solution and a 5 per cent suspension made from the packed cells in this solution.

Sensitized sheep cells: This suspension was prepared by mixing equal parts of the 5 per cent cells with a dilution of antish sheep cell amboceptor made up in 0.85 per cent salt solution to represent two units of amboceptor for each 0.1 cc. of the 5 per cent cell suspension.

Complement: Fresh guinea-pig serum was diluted with 0.85 per cent salt solution so that 0.1 cc. contained two complement units.

Blood platelets: The blood platelets were separated from citrated sheep's blood by centrifugalizing at low speed for twenty minutes. The supernatant fluid which contained the platelets was then removed and centrifugalized at high speed and the sedimented platelets washed three or four times in 0.85 per cent salt solution, centrifugalizing each time at high speed for thirty minutes.

Citrate and oxalate solutions: These solutions were prepared by adding 2 per cent of each of these salts to 0.5 per cent sodium chloride solutions.

The experiments

Experiment 1. Experiment demonstrating the agglutinative and lytic action of calf sera for 5 per cent sheep cells alone and also in the presence of complement and for sensitized sheep cells.

0.3 cc. quantities of the serum from four calves both before and after inactivation in the water-bath at 56°C. for one-half hour were mixed with (1) 0.1 cc. of five per cent sheep cells; (2) 0.1 cc. of 5 per cent sheep cells and 0.1 cc. of complement; (3) 0.2 cc. of sensitized sheep cells.

With one of the calves, No. III, similar mixtures were made containing 0.2 cc. and 0.1 cc. of the calf serum to control experiment 5 and protocol 4. These mixtures were placed in the water bath at 37.5°C. for one hour when the reactions were read and recorded.

Results. The active serum of all four of the calves tested produced a strong agglutination of sheep cells, sensitized, unsensitized, and 5 per cent cells in the presence of complement. The serum of calf No. III, showed considerable lytic activity also. The sera of the other three calves showed slight or no lytic action. The sera after inactivation showed marked diminution in agglutinative effect and partial inhibition in lytic action.

Protocol 1. Protocol 1 demonstrates the agglutinative and lytic action of active and inactivated calf sera for sensitized and for unsensitized sheep cells and for the latter in the presence of complement

CALF SERUM	5 PER CENT SUSPENSION OF SHEEP CELLS 0.1 cc.		5 PER CENT SUSPENSION OF SHEEP CELLS 0.1 cc. COMPLEMENT 0.1 cc.		2.5 PER CENT SUSPEN- SION OF SENSITIZED SHEEP CELLS 0.2 cc.	
	Agglutina- tion	Hemolysis	Agglutina- tion	Hemolysis	Agglutina- tion	Hemolysis
I. Active 0.3 cc....	++++	None	++++	None	++++	None
I. Inactive 0.3 cc...	+	None	+	None	+	None
II. Active 0.3 cc....	++++	None	++++	None	++++	None
II. Inactive 0.3 cc...	+	None	+	None	+	None
III. Active 0.3 cc....	++++	Partial	++++	Strong	++++	Partial
III. Active 0.2 cc....	++++	Slight	++++	Partial	++++	Slight
III. Active 0.1 cc....	None	Slight	++++	Partial	+	Slight
III. Inactive 0.3 cc...	+	None	+	Partial	+	None
IV. Active 0.3 cc....	++++	Slight	++++	Slight	++++	Slight
IV. Inactive 0.3 cc...	None	None	None	Very slight	None	None

++++ = strong agglutination.

+ = partial agglutination.

Experiment 2. Experiment demonstrating the clotting effect of blood platelets upon calf serum.

The blood platelets obtained from 100 cc. of sheep's blood were added to 3 cc. of the active serum from calf No. III. The mixture was kept at 37.5°C. for two hours and then removed to the cold room over night.

Results: Clot formation was observed after several minutes, the serum at first presenting a solid appearance and then gradual clumping of the suspended cellular elements and retraction of the clot.

Experiment 3. Experiment demonstrating that calf serum after treatment with blood platelets and removal from the resulting secondary clot no longer possesses agglutinative and lytic action for sheep cells, either sensitized, unsensitized, or unsensitized in the presence of complement.

0.3 cc. quantities of treated active serum from calf No. III were added to (1) 0.1 cc. of five per cent sheep cells; (2) 0.1 cc. of five per cent sheep cells and 0.1 cc. of complement; (3) 0.2 cc. of sensitized sheep cells.

The mixtures were placed in the water-bath at 37.5°C. for one hour when the reactions were read.

*Results:*² No agglutination or hemolysis was observed in any of the tubes while control tests with untreated calf serum containing the clotting elements showed strong agglutination and hemolysis. (See Protocols 1 and 2.)

Protocol 2. Protocol 2 demonstrates the loss in agglutination and hemolytic properties of calf serum removed from the clot formed by treatment of the serum with blood platelets

CALF SERUM 0.3 CC. AFTER REMOVAL FROM SECONDARY CLOT INDUCED BY ADDITION OF BLOOD PLATELETS	5 PER CENT SUSPENSION SHEEP CELLS 0.1 CC.		5 PER CENT SUSPENSION SHEEP CELLS 0.1 CC. COMPLEMENT 0.1 CC.		2.5 PER CENT SUSPENSION SENSITIZED SHEEP CELLS 0.2 CC.	
	Agglutination	Hemolysis	Agglutination	Hemolysis	Agglutination	Hemolysis
III. Active....	None	None	None	None	None	None

See Protocol 1 for control tests upon untreated serum of calf No. III.

Experiment 4. Experiment demonstrating the inhibition of agglutination and partial inhibition of hemolysis by the anti-coagulants, sodium citrate and sodium oxalate.

² Identical results were obtained with the sera of the other 3 calves.

0.2 cc. quantities of sodium citrate and sodium oxalate were added separately to mixtures of 0.3 cc. of active serum from calf No. III with (1) 0.1 cc. of five per cent sheep cells; (2) 0.1 cc. of five per cent sheep cells and 0.1 cc. of complement; (3) 0.2 cc. of sensitized sheep cells.

The mixtures were placed in the water-bath at 37.5°C. for one hour, when they were read.

Results: No agglutination was observed with any of the mixtures but slight hemolysis occurred in the mixture containing five per cent cells, citrate solution and complement, while the control tests in the absence of the anti-coagulants showed strong agglutination and hemolysis as indicated in Protocol 1.

Protocol 3. Protocol 3 demonstrates the inhibition of the agglutinative and lytic properties of calf serum for sheep cells by the anti-coagulants, sodium citrate and oxalate

CALF SERUM 0.3 CC.	5 PER CENT SUSPENSION SHEEP CELLS 0.1 CC.		5 PER CENT SUSPENSION SHEEP CELLS 0.1 CC. COMPLEMENT 0.1 CC.		2.5 PER CENT SUSPENSION SENSITIZED SHEEP CELLS 0.2 CC.	
	Agglutination	Hemolysis	Agglutination	Hemolysis	Agglutination	Hemolysis
III. Active plus 0.2 cc. citrate solu- tion	None	None	None	Slight	None	None
III. Active plus 0.2 cc. oxalate solu- tion	None	None	None	None	None	None

For control tests in the absence of anti-coagulants see Potocol. 1

Experiment 5. Experiment demonstrating the partial inhibition of the lysis of sheep cells by calf serum when the blood platelets and leucocytes are removed from the sheep cell suspension.

For this experiment sheep's blood was collected in citrated salt solution and washed six times centrifugalizing at low speed and removing the upper layer of cells with the supernatant fluid at each washing.

Five per cent and sensitized suspensions were then prepared from cent these cells as described under "reagents".

0.3 cc., 0.2 cc. and 0.1 cc. of the active serum from calf No. III were then added to (1) 0.1 cc. of 5 per cent cells; (2) 0.1 cc. of 5 per cent cells and 0.1 cc. of complement; (3) 0.2 cc. of sensitized cells.

The mixtures were placed in the water-bath at 37.5°C. for 1 hour when they were read.

Results: Strong agglutination was observed with the 0.3 cc. quantities of calf serum. Some inhibition of agglutination occurred with smaller quantities of serum. No diminution in the lytic action was seen as compared with the controls. See Protocols 1 and 4 for comparison of the lytic and agglutinative action with the ordinary cell suspension.

Protocol 4. Protocol 4 demonstrates the effect upon the agglutinative and lytic action of calf serum for sheep cells, of removing the blood platelets and leucocytes from the cell suspension

CALF SERUM	5 PER CENT SUSPENSION SHEEP CELLS—PLATELETS OUT—0.1 cc.		5 PER CENT SUSPENSION SHEEP CELLS—PLATELETS OUT—0.1 cc. COMPLEMENT 0.1 cc.		2.5 PER CENT SUSPENSION SENSITIZED SHEEP CELLS— PLATELETS OUT—0.2 cc.	
	Agglutina- tion	Hemolysis	Agglutina- tion	Hemolysis	Agglutina- tion	Hemolysis
III. Active						
0.3 cc.	++++	Partial	++++	Strong	++++	Partial
0.2 cc.	++	Slight	++++	Partial	++++	Slight
0.1 cc.	None	Slight	++++	Partial	None	Slight

For control test with cell suspension containing blood platelets and leucocytes see Protocol 1.

++++ = strong agglutination.

++ = moderate agglutination.

SUMMARY

1. The sera of four calves were found to agglutinate sheep cells. One of these sera produced strong hemolysis of sensitized sheep cells and partial hemolysis of unsensitized cells. The serum from one of the other calves produced slight hemolysis of both sensitized and unsensitized cells.

2. The addition to the active calf serum of cytozyme in the forms of blood platelets caused the formation of a second clot.

3. The serum from this second clot did not agglutinate or hemolyze sheep cells, either sensitized, unsensitized, or unsensitized in the presence of complement.

4. The addition of anti-coagulants, sodium citrate or oxalate, to the mixtures of active calf serum and cells prevented the agglutination of the latter and completely inhibited their hemolysis except in one instance in which the citrate was mixed with

active serum, unsensitized sheep cells and complement. Only partial inhibition was obtained with this mixture.

5. Removal of the blood platelets from the sheep cell suspension reduced but did not remove the agglutinative action of the calf serum and did not affect the hemolysis.

CONCLUSIONS

The agglutination of sheep cells by calf serum is caused by the formation of fibrin induced by the reaction between cytozyme present in the sheep cell suspension and the other elements necessary for clot formation which are present in the serum. The fibrin forms around groups of cells as centers of coagulation and precipitates out carrying down the suspended blood cells.

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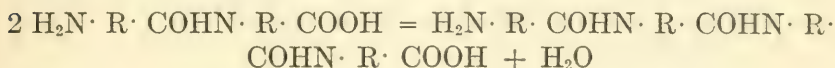
IMMUNOLOGICAL EXPERIMENTS WITH DENATURED AND INSOLUBLE PROTEINS

CARL L. A. SCHMIDT

*From the Department of Biochemistry and Pharmacology of the University of
California, Berkeley*

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The studies of Chick and Martin (1), Robertson (2) and others (3) have given us a clear conception of the mechanism involved in the heat coagulation of proteins. The phenomenon involves two distinct processes: (a) denaturation, (b) agglutination. The first step is a process of dehydration involving the loss of water from the elements contained in the terminal $-NH_2$ and $-COOH$ groups of two or more protein molecules.



The reaction¹ does not take place instantaneously but proceeds with a definite velocity (provided the acidity is kept constant), the relation between time and amount of denatured protein being characteristic of the monomolecular type of reaction. The agglutination of the denatured protein requires an optimum acidity or salt concentration and since the process is very rapid, the rate of flocculation of protein heated under optimum conditions is dependent wholly on the slower process; viz., denaturation. The denatured protein molecules in the process of agglutination form particles of large magnitude which appear as masses of insoluble protein. Under ordinary conditions the process is irreversible, but if, as shown by Corin and Ansiaux (4), when flocculation becomes apparent, the solution be cooled and rapidly

¹For other possible reactions see Robertson, T. B., *The Physical Chemistry of the Proteins*, New York and London, p. 127.

shaken, solution is effected. In certain instances the process of heat denaturation is easily reversible. Thus Osborne (5) has shown that the marked opalescence which appears on heating solutions of caseinates disappears on cooling and reappears when the solution is heated. Heating protein in the dry state usually does not lead to insolubility although denaturation takes place. Water is probably lost from the elements contained in the terminal groups of the individual protein molecules and ring compounds of the following type are probably formed.



These compounds do not polymerize and hence are not rendered insoluble.

From an immunological standpoint the effect of heat on the properties of proteins is important. The early workers (6) noted that proteins can be heated to a relatively high temperature without losing the power of sensitizing animals to a subsequent injection of native protein but that the intoxicating power of the heated protein is markedly affected. They attempted to use these facts as a basis for an hypothesis that there exists in proteins a sensitizing and an intoxicating fraction. Wells (7) found that coagulation of protein by alcohol or denaturation by heating the solution to 100°C. does not impair the ability of the protein to act as antigen for the anaphylaxis reaction provided in the treatment the solubility of the protein is not lost. If, however, the protein is coagulated by heat it can still sensitize guinea-pigs for a subsequent dose of native protein but the power of inducing anaphylaxis in animals sensitized to native protein is almost if not wholly lost. Thus a solution of egg albumin which had been heated to 100°C. for fifteen minutes sensitized guinea-pigs fatally to a subsequent dose of the unheated antigen but only slight symptoms were produced in animals which had been sensitized to native egg albumin when given a second dose of the same protein solution which had been heated to 90°C.

W. A. Schmidt (8) carried out extensive investigations on the influence of heat on the antigenic properties of blood serum.

He found in agreement with Loeffler (9) that the dry protein may be heated to 130°C. and still be precipitated by immune sera for the native protein. In solution the protein is more susceptible to the influence of heat. Serum when heated above 90°C is no longer precipitated by antisera for the unheated serum and precipitating sera when heated to 70°–75°C. loses its power of producing precipitates. (See also Obermayer and Pick 18.) Schmidt also carried out experiments to determine the influence of heat on the ability of heated proteins to give rise to precipitating antibodies when repeatedly injected into rabbits. The sera of the animals immunized to 70°C. serum were found to react better with the homologous antigen than with the native serum; curiously, however, on continued immunization with the heated serum, the sera of the immune animals were found to react also with native serum without losing the ability to give precipitates with heated serum.

An analysis of the influence of heat on proteins shows that the process follows one of the following types of reaction: (a) denaturation by heating the protein in the dry state, a process which does not render the protein insoluble unless the temperature is excessively high. (b) Denaturation by heating a solution of the protein, a process which in certain instances (solution of the caseinates) is reversed on cooling the protein solution. (c) Denaturation of the protein in solution with or without subsequent agglutination. In certain instances (solutions of egg albumin) the solution is rendered opaque but flocculation does not take place; under optimum conditions of acidity (isoelectric point) and salt concentration the protein is rendered insoluble. The process is apparently irreversible on account of the hysteresis in the heterogenous system. (d) Hydrolysis of the protein to yield split products, a process which involves a fundamental change in the chemical makeup of the protein molecule. This process has a marked effect on the antigenic property of the protein (10) and depends largely on the degree of splitting. Excluding the latter process and the type of heat denaturation which is reversible two factors remain whose influence on antigenic property must be considered; viz., denaturation *per se*

and loss of solubility through coagulation. With the exception of the experiments reported by Landsteiner (19, 20) and his collaborators, no experiments to determine the influence of these factors on the production of fixation antibodies appear to have been carried out and hence this reaction was used in our experiments as a criterion to determine the presence of immune bodies.

Rabbits were immunized by injecting (alternately subcutaneously and intraperitoneally) at regular intervals, 100 mgm. doses of egg albumin prepared according to the method of Hopkins and Pinkus (15) as modified by Osborne (16) and used recently in this laboratory by Haas (17).

Rabbit R was given 600 mgm. of egg albumin which had been heated (dry) to 110°C. for one hour. The solubility of the preparation was not materially affected.

Rabbit M and H were given each 600 mgm. of egg albumin which had been heated in solution to 100°C. for 15 minutes. The initial reaction was adjusted to pH 7.1. The egg albumin was not coagulated although the solution became opaque.

Rabbit F was given a suspension of 600 mgm. of coagulated egg albumin which was prepared by boiling the egg albumin solution for fifteen minutes.

Fixation experiments were carried out with the inactivated sera of the immunized animals and the following antigens, in a dosage of 0.1 cc. of a 0.2 per cent (in saline) solution, were used:

1. Egg albumin heated to 110°C. for one hour.
 2. Native (unheated) egg albumin, reaction pH 7.2.
 3. Native (unheated) egg albumin, reaction not adjusted.
 4. Egg albumin solution heated to 100°C. for fifteen minutes.
- Initial reaction, pH 7.2. No coagulum was produced.

The results of the fixation experiments are shown in the following table.

Results of fixation experiments with egg albumin

ANTIGEN	SERA			
	F	H	M	R
1	0.4 cc. of 1:50	0.4 cc. of 1:50	0.3 cc. of 1:50	0.4 cc. of 1:10
2	0.4 cc. of 1:50	0.1 cc. of 1:50	0.1 cc. of 1:10	0.3 cc. of 1:50
3	0.4 cc. of 1:50	0.1 cc. of 1:50	0.4 cc. of 1:250	0.3 cc. of 1:50
4	0.4 cc. of 1:50	0.1 cc. of 1:10	0.1 cc. of 1:10	0.1 cc. of 1:10

NOTE: The figures indicate the minimum amount of the given dilution of serum with which fixation was obtained.

As seen from the table all reactions are positive, indicating the presence, in the sera of the immunized animals, of fixation antibodies. No specificity with respect to the antigen is shown. The immune sera react with egg albumin irrespective of whether the latter is native or denatured provided solubility was not lost. The results are not unexpected when it is recollected that denaturation does not involve any fundamental change in the chemical makeup of the protein molecule. Moreover experiments carried out by Gay and Robertson (11) in which they found that paranuclein, a casein split-product, gives rise, in immune animals, to antibodies not only for itself but also for casein, indicate that a limited change in the chemical makeup may be produced in the protein molecule without loss of ability to induce antibody production for the original protein molecule.

The question of solubility deserves further consideration. In our experiments it is not certain that coagulation led to complete insolubility. To throw further light on the question two rabbits were immunized with a suspension of finely ground casein which had been repeatedly washed with dilute acetic acid, water, alcohol and ether. The antigen was injected intraperitoneally in 200 mgm. doses and each animal received six injections. Fixation tests, using a neutral solution of sodium caseinate as antigen, were carried out with the inactivated sera of the two animals and in each instance positive tests were obtained. The fixation tests were controlled by using egg albumin as antigen and negative results were obtained. Skin tests were also carried out on these animals by injecting intradermally 4 mgm. each of casein,

egg albumin, ovomucoid, and paranuclein. Typical reactions were obtained with casein and paranuclein but not with the other proteins.

These experiments do not indicate the mechanism whereby anti-bodies are produced when an insoluble protein is injected. It is a well-known fact that particulate matter when introduced into the peritoneal cavity appears in the blood stream in a very short time (12) and it is possible that with sufficient time solution is eventually effected. Moreover it should be recollected that insolubility of a protein, as with other substances, is a relative matter. Casein is usually considered to be insoluble in water and blue litmus paper does not turn red when dipped into water which is used to wash casein. If, however, the litmus paper comes in contact with the particles of suspended casein it is reddened indicating traces of a surface film of soluble casein which adheres to the suspended casein particle (13). The difference in the ability of an insoluble protein to give rise to immune bodies and also to sensitize and its non-ability to produce anaphylaxis lies in the time factor. For the latter reaction solubility (13) as well as a sufficient concentration of the antigen is essential.

SUMMARY

To study the influence of denaturation, coagulation and insolubility on the production of fixation antibodies, rabbits were immunized respectively against the following antigens: Egg albumin heated (dry) to 110°C., egg albumin heated in solution, to 100°C. without coagulation, egg albumin coagulated by heat. The sera of these animals reacted equally with the native (unheated) and with the denatured antigen. Using sodium caseinate as antigen, fixation antibodies were demonstrated in the sera of rabbits which had been immunized by injecting a suspension of washed casein. The theoretical considerations are discussed.

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ON THE ESSENTIAL IDENTITY OF THE ANTIBODIES

HANS ZINSSER

*Department of Bacteriology, College of Physicians and Surgeons,
Columbia University*

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Since Ehrlich's first classical analysis of antibodies, it has been a generally accepted conception of immunity that agglutinins, precipitins, sensitizers, bacteriolysins, hemolysins, or the so-called amboceptors, opsonins and the anaphylactic antibodies are separate substances formed in the animal body, often in response to treatment with a single antigen. Kraus, in the first edition of Kolle and Wassermann's Handbook, summarizes this point of view unambiguously in the following words:

Just as the bacterial body contains a variety of different antigens, so we may assume that animal protein is made up of a large number of different antigenic elements. If the animal body is treated with such substances and finds corresponding receptors, there results the formation of a variety of qualitatively different antibodies. . . .

When Gengou, in 1902, noted that alexin or complement was fixed when a precipitating antiserum was added to its homologous antigen, he interpreted this as meaning that, in addition to precipitins, the antiserum contained other antibodies—the "albuminolysins." To be sure, Gay and Moreschi showed that the fixation of alexin was chiefly a property of the precipitate which was formed, but this was regarded as signifying that the protein sensitizers were mechanically carried down during the precipitation. Ehrlich commenting upon this in 1910, says:

It seems reasonable to assume, in accordance with Gengou's first explanation, that the property of binding the complement is exercised

by the albuminous bodies sensitized with a specific amboceptor and, just as when immunizing with cells agglutinins and amboceptors are formed, so also when immunizing with dissolved albuminous bodies two kinds of antibodies are formed, precipitins and amboceptors.

To be sure the idea that agglutinins and precipitins might represent one and the same antibody has been more or less prevalent since their first observation. It originated, we believe, with Paltauf and was expressed as a widely accepted view by v. Eisler in a review of precipitin reactions published in 1909. But, in regard to identification of other antibodies with these two, there has been either a complete disregard of such a possibility or, when suggested, it has been thrown out of consideration because of the frequent and complete lack of quantitative parallelism between the curves of the various antibody functions in one and the same serum.

The idea of such a possible identity, however, has cropped up again and again and, in our own work, has gradually forced itself upon us so insistently that we have thought it important to bring it forward again.

The fundamental idea was expressed quite definitely in 1908 by Bail and Hoke who made an extensive study of the bacteriolytic, precipitating and agglutinating actions of normal beef serum and immune rabbit serum upon cholera spirilla. They clearly expressed the opinion that there were no separate bacteriolytic, precipitating or agglutinating antibodies in these sera; that the essential fact was the existence, in the sera exerting these effects, of a single antibody which united with the bacterial substances and that the various reactions which followed were the results of the conditions under which the different observations were made. Although a similar idea, on somewhat less valid evidence, had been expressed by Bürgi, and although Bail and Tsuda followed out the thought in subsequent publications, the view has made little actual headway, in spite of much corroborative, though scattered, evidence.

In order that there may be no ambiguity as to just what is meant by what we call the "unitarian" view of antibodies, let us begin by formulating it clearly.

By such a conception of antibodies, we do not, of course, imply that a complex cell like, for instance, the typhoid bacillus can give rise to one variety of antibody only. There may be formed a specific sensitizing antibody against the major chemical constituent, and other sensitizers against other antigenic substances enclosed in the same cell body or contained in the same antigenic solution. But we do mean that, were we working with a single antigen, in a pure state, one variety of antibody only would be produced. This would be present in the form of a serum constituent specifically capable of uniting with the antigen. As a result of the union, the antigen is altered in its physical, and perhaps, to some extent, in its chemical behavior. The resultant reactions which may be observed with this sensitized antigen (agglutination, precipitation, complement fixation, bactericidal phenomena, bacteriolysis, opsonization or sensitizing effects in the anaphylactic sense) would be determined not by differences in the nature of the antibodies with which the antigen had united, but rather by the physical state of the antigen itself, the nature of the cooperative substances (alexin, leucocytes, tissue cells) and by the environmental conditions under which the observations are made.

Thus, if the antibody comes in contact with a very finely divided antigen, as in a bacterial extract or in, let us say, horse serum, if electrolytes are present and perhaps other necessary physical factors furnished by the presence of serum, etc., precipitation occurs.

When we are dealing with whole bacteria of relatively large mass and correspondingly small surface exposure, agglutination is the result, and quantitative parallelism with the precipitin reaction is not to be expected because of the much greater dispersion of the antigen in the latter test.

When alexin is present, complement fixation or hemolysis or bactericidal effects result, since the changes produced by the sensitization have now permitted union with the complement.

When there are leucocytes present the union makes possible the phagocytosis of the antigen, and when the antibody is absorbed by the cells of an animal, anaphylactic "sensitization" occurs.

As we have stated, the earlier opposition to such a view was largely based upon lack of quantitative parallelism between agglutination and precipitation curves on the one hand, and bactericidal or protective antibody curves on the other, and this in spite of the relative inaccuracies which biological measurements of this nature imply.

In appraising such objections, however, we must not forget that agglutination and precipitation are actually only secondary phenomena, after the union of antigen and antibody has taken place, and are dependent upon a great many environmental factors which may not, to the same degree, influence phenomena in which alexin, the leucocyte or the body cells of animals are involved. We need only to point out the frequently observed alterations and diminutions of the agglutination and precipitating powers of sera by heat. Heating antibacterial sera even to 56° to 60°C. will often materially diminish their precipitating effects for bacterial extracts, an observation which is entirely analogous to the influence of heating (to 60° to 70°C.) on the flocculating effects of serum for various colloidal suspensions, like arsenic trisulphide, etc. In addition to this, the flocculation reactions depend upon the presence and the concentration of electrolytes, upon reaction, upon mutual relations of concentration, and perhaps upon viscosity. Moreover, the suspension equilibrium of the sensitized antigen must to some extent depend upon the varying factor of the inactive serum constituents carried into the union with the antibody. For we know that in precipitation reactions the bulk of the precipitate comes from the serum, and yet relatively protein-free antibodies can be split off from such a complex (Gay and Chickering, Chickering, Huntoon), conditions which prove that, in the union, much inactive protein substance is carried along, which inevitably must influence reactions of flocculation. Indeed, Tulloch has suggested that these "presumably inactive constituents" may even protect the united antigen-antibody complex from flocculation, a conception which we believe explains the "agglutinoid" phenomena.

It is not to be wondered at, therefore, that agglutination and precipitation curves should not run parallel with the curves

of other antibody functions. And it is worth noting that, in regard to such lack of parallelism, while it has frequently been noted that agglutinating and precipitating functions were often weaker than other antibody effects, or even absent entirely in such sera, it has rarely been observed that they were powerfully and specifically present when other effects were lacking.

In addition, however, to these purely quantitative objections to the "unitarian" conception, other arguments have been advanced, such for instance as those of Gay and Stone who observed, with cholera extracts and cholera sera, that the formation of precipitates removed little or no lytic substance from the supernatant fluid. As against this, however, we have the experiments of Bail and Tsuda who found that from specific cholera precipitates, obtained as above and injected into the peritoneal cavities of guinea pigs, lytic and bactericidal antibodies were liberated and effective in producing a Pfeiffer reaction.

We must confess that such failure of quantitative removal of antibodies from the supernatant fluid, after agglutination and precipitation reactions had been carried out with these sera, was our own experience with typhoid serum, and there are certain points here that require further investigation. But when one considers, as we have stated above, that the amount of sensitizer necessary to bring about an agglutination or precipitation may be very slight, even in the presence of an excess of antigen, and that from such agglutinated or precipitated bacteria or bacterial extracts dissociation can take place; and if we then further consider the great inaccuracies coincident to the quantitative determination of bactericidal and bacteriolytic effects, such experiments lose much of their weight. One need only recall such experiments as those of Topfer and Jaffe who found absolute lack of uniformity between plating tests and Pfeiffer phenomenon in determining bactericidal substances in one and the same sera. There are too many and uncertain secondary factors necessary in all these purely biological methods to give one much confidence in any reasoning based purely on quantitative results.

More recently the growing interest in the purification of antibodies by dissociating them from antigen-antibody unions

has again led to attempts to separate antibodies in the pure state. Landsteiner in his early work on dissociation makes no particular point of this phase of the problem. Gay and Chickering found that protective antibodies could be extracted from specific precipitates with dilute sodium carbonate, but interpreted this as a mechanical absorption of the protective bodies during precipitation. Huntoon succeeded in sensitizing pneumococci, heavily, and then, by treating them with slightly alkaline salt solution at 55°C. dissociating from them protective antibodies in considerable concentration, and in solutions which are practically protein-free. With these antibody solutions he has been able to protect mice. It is not our function to go into the experiments of Huntoon more extensively, since these are all published or in the process of publication. The important point for us is the fact that these highly protective antibody solutions fail to agglutinate pneumococci in a large number of tests made by him, although they possessed distinct protective powers.

Having considered some of the more important objections, let us see what may be said in favor of the "unitarian" view apart from its simplicity.

The identification of agglutinins with precipitins should hardly require much argument since both are specific flocculation reactions between a serum and the same antigenic substance, depending upon analogous environmental conditions. That they should differ from each other quantitatively is to be expected from the fact that in one case the antigen is present in relatively large masses and in the other case it is finely dispersed.

That there is at least a strong likelihood that precipitins and bactericidal and protective antibodies may be identical is indicated by the experiments of Bail and Tsuda who dissociated bacteriolytic cholera antibodies from precipitates obtained with cholera extracts and normal beef serum.

Bail and Hoke obtained similar results not only with normal beef serum, but with immune rabbit serum, though in the latter case somewhat less sharply. The latter point is important because the conglutination question is involved in their experiments with normal serum for they obtained much better precipitates

with the normal beef serum in its active state. However, since sensitization with an antibody is necessary for the congrutinin effect, the principle of Bail and Hoke's experiment in regard to the liberation of bacteriolytic sensitizer from precipitated cholera extracts remains the same.

Also the identity of precipitins and protective antibodies is very strongly suggested by the experiments of Gay and Chickering, inasmuch as they succeeded in extracting protective pneumococcus antibodies from specific precipitates, and the identity of the two is at least likely as mere mechanical carrying down with the precipitate.

The probable identity of precipitins with complement fixing antibodies is indicated by the work of Dean and by many observations of our own.

In 1912 Dean, analyzing the relationship between alexin fixation and precipitation, concluded that the proportions of antigen and antibody which favor rapid and complete precipitation do not favor complete alexin fixation. He did not believe that the two reactions followed a parallel course, but said that he thought they represented two phases of the same reaction, a "flocculation representing the first stage of a change that can be recognized in its early stage by complement fixation." In 1912 and 1913 we were engaged in a similar analysis from which we came to the conclusion that there was no need for assuming that the antibodies which were involved in the fixation of the alexin were essentially different from those that brought about the precipitation.

Studying the relationship between precipitin reaction and complement fixation in the reactions between sheep serum and anti-sheep rabbit serum, like so many other observers, we found that amounts of antibody which did not precipitate would, nevertheless, produce complement fixation reactions in such mixtures and this, of course, is the basic principle of the forensic complement fixation, introduced and described practically by Neisser and Sachs. In order to eliminate the possibility of non-specific mechanical carrying down of the complement fixing antibody, we carried out experiments with complement fractionation, and found that the fixation by the precipitates and by the supernatant

fluids in such mixtures took place in the same way that fixation by sensitized cells occurs; namely, that the precipitates first fixed end-piece and mid-piece, secondarily. From this and other experiences, we expressed the conclusion at that time that "there is but one variety of specific sensitizer," and that "the visible precipitation is merely secondary, occurring because of the colloidal nature of the reacting bodies under quantitative and environmental conditions which favor flocculation." Furthermore, the experiments of the last ten years have shown again and again that the physical state, the formation of precipitates and the changes in sizes and perhaps in electrical conditions of the substances in serum reactions are intimately related to the fixation of alexin. The recently developed observations on the Wassermann reaction, the Vernes test and the Sachs-Georgi reaction now successfully employed in our laboratory are all evidence in this direction.

That the anaphylactic passive sensitizing effect of sera was found to be quantitatively proportionate to the precipitin contents of these sera, was shown by Doerr and Russ in experiments which seem completely to justify the identity of precipitating and anaphylactic antibodies suggested by Friedberger in 1908.

In regard to the dissociation experiments of Huntoon, some of whose solutions have been kindly sent to us by Dr. Huntoon, himself, we have, indeed, found that these solutions exert considerable protective effect upon mice and fail either entirely, or almost entirely to produce agglutination "*in vitro*" of the pneumococcus against which they protect.

This would seem a potent argument against the "unitarian" idea. However, it must be considered that the "protein-free" antibody of Huntoon is physically, and, therefore, in its agglutinating and precipitating functions quite a different substance from the original antibody in the unchanged serum, which in its native state is inactive, associated with the pseudo-globulin substances which it carries down with it into precipitates. For, as we have stated before, it is of course known that most of the substance which comes down in precipitin reactions is derived from the immune serum. Moreover, by, to some extent, restoring

the Huntoon antibodies to their original environment in the circulation of an animal, we were able to observe powerful agglutination. We repeated, with these substances, the experiment on the mechanism of serum protection against pneumococci in rabbits, carried out by Bull in 1915. A rabbit of approximately 1200 grams was intravenously injected at 5 p.m. with about 8 cc. of a broth culture of pneumococcus I of relatively low virulence. At 10 a.m. the next morning the rabbit was very sick, and a heart's blood puncture was done which showed numerous pneumococci evenly distributed through the blood stream. Ten cubic centimeters of Huntoon's material was injected intracardially in this animal, the needle was withdrawn and about two minutes later blood was taken from the heart and smeared. Similar punctures were done five minutes later and fifteen minutes later. As will be seen from the attached illustration, smears from the heart's blood taken two minutes later showed the pneumococci in the blood in clumps of varying sizes, with very few small clumps of two and three, and a very few individual organisms. But the large majority of the organisms were now in clumps of 10 or more members. Smears after five minutes showed a few clumps and a few individual organisms, and after fifteen minutes there were very few single organisms and no clumps, but it was then hard to find organisms, whereas before injection every field showed ten or more.

We did not succeed in thus restoring the agglutinative functions of Huntoon's materials in all cases. But the reason for this was revealed, we believe, by experiments carried out for us by Mrs. Parker. She found that the Huntoon substances, produced by dissociating sensitized pneumococci in dilute sodium bicarbonate solutions at 55°C. had a reaction of pH 8.8 to pH 9.4. When powerfully agglutinating pneumococcus sera were subjected to similar treatment at similar reactions, their agglutinating powers were largely lost and but partially restorable by neutralization. It would seem that these various considerations should serve materially to weaken the validity of objections to the "unitarian" theory based upon the separation of antibodies by dissociation experiments.

Furthermore, observations recently made by Coulter seem to us to have considerable bearing upon this question in indicating that agglutination and the sensitization to hemolysis of red cells, are both due to the action of one substance. Coulter found that "the optimum hydrogen ion concentration for the agglutination of sensitized cells (rabbit-antisheep sensitizer), in a salt-free medium, occurs at pH 5.3 which corresponds with the optimal point for the precipitation of the serum-globulin itself." The optimum for precipitation of serum-globulin, in which the immune bodies are carried, is stated by Rona and Michaelis as pH 5.2 and the iso-electric point for typhoid immune bodies as pH 5.4. This reaction is more alkaline than that which is optimal for the agglutination of normal sheep cells in saccharose solution, so that Coulter concludes, as far as agglutination is concerned, the behavior of sensitized cells is closely related to the properties of the immune serum. We quote directly from a personal communication from Coulter as follows:

Furthermore, an equilibrium has been found to exist between the amount of hemolytic sensitizer free and that combined with cells, the amount which combines at a pH of 5.3 being at the maximum, and approximating 100 per cent.

These observations indicate that the hydrogen ion concentration at which the agglutination of sensitized cells is most perfect corresponds with the iso-electric point of that part of the serum which makes the antibodies and also corresponds with the point at which the largest amount of hemolytic sensitizer is absorbed by the red cells. This, to our mind, would be strong evidence in favor of identity of the hemolytic sensitizer and the agglutinin.

We do not wish by any means to convey the impression that we consider the "unitarian" view as absolutely and rigidly proven. We do believe, however, that the denial of such a view necessitates the assumption that the injection of a pure antigen calls forth five or six fundamentally different reactions on the part of the tissue cells, a theory which would be justified only on the basis of incontrovertible proof.

If there is nothing further to be said in favor of the "unitarian" view, one might at least wait for further evidence before one tried to deny an existing view, however complicated. But there is much to be said in favor of it and evidence in this direction is accumulating.

We have believed in the probable truth of the "unitarian" view for a considerable number of years, with sufficient conviction to teach it as the most likely state of affairs. And while we cannot prove it in all the ramifications of the difficult experimental problems involved, we believe it has gone far enough certainly to throw the burden of proof upon those who still cling to the separation and the conception of separate structure for agglutinins, precipitins, bacteriolysins, etc.

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STUDIES ON ACUTE RESPIRATORY INFECTIONS

VIII. A STUDY OF THE CULTURAL AND SEROLOGICAL RELATIONSHIP OF HEMOLYTIC STREPTOCOCCI ISOLATED FROM INFLAMMATORY CONDITIONS OF THE RESPIRATORY TRACT¹

EUGENIA VALENTINE AND LUCY MISHULOW

From the Bureau of Laboratories, Department of Health, New York City

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This study is part of the general investigation of the diseases of the respiratory tract. The purpose of this study was to determine whether or not a dominant variety of hemolytic streptococcus could be isolated from the cases of acute colds and influenza studied.

The rôle played by the hemolytic streptococcus in a considerable percentage of the fatal pneumonias in 1918 has stimulated many investigators (1), (2), (3), (4), (5), to study the incidence of this organism in the secretions of the nose, throat and in the sputum of normal persons and in conditions of respiratory disease. They also studied the fermentative reactions in an attempt to correlate the fermentative types with the character of the respiratory condition.

The work of these investigators is of interest primarily in that it indicates that the streptococcus hemolyticus is frequently present in the throat, especially if the tonsils be large and cryptic. As to the correlation of definite fermentative types with the conditions in which they were found, the results are not altogether concordant. These results bear only indirectly on

¹ One of a series of studies carried out under the direction of William H. Park, Anna W. Williams and Charles Krumwiede. The previous studies in this series were reported in the *Journal of Immunology*, vi, no. 1, January, 1921. This investigation was made possible by a grant of money from the Metropolitan Life Insurance influenza fund for a part of the expense.

our problem, as the fermentative reactions give only broad primary groups, which groups are further divisible by immunological reactions.

Several investigators have studied the immunological reactions of the streptococci isolated from respiratory diseases. Most noteworthy is the work of Dochez, Avery and Lancefield (6) who studied the immunological relationship of hemolytic streptococci especially from cases of pneumonia, many of which were secondary to measles. The material was obtained mainly from the military establishments about Fort Houston. These authors added to the evidence already available that fermentative differences are strongly indicative of immunological differences, whereas fermentative similarity is not necessarily paralleled by immunological similarity. The earlier studies, especially those of septic sore throat (Smith and Brown (7), Krumwiede and Valentine (8) had indicated this.

In our attempt to find a dominant strain of streptococcus hemolyticus in this series of cases, the plan was to study first the general biological characteristics in order to determine what groups were separable by fermentative and hemolytic reactions. The members of these groups were then studied immunologically by agglutination, and where strains were seemingly alike, agglutinin absorption was employed as the final criterion of identity or non-identity.

The cultures studied were obtained from the nose, nasopharynx or throat in acute colds, rhinitis and pharyngitis, and from the more severe influenzal conditions. A few pleurisy and mastoid cases are included, as well as normal controls. In many instances several examinations were made from the same case both during illness and after recovery.

The method of isolation of the organisms for this study was as follows:

A sterile cotton swab was rubbed over the surface of the mucous membrane of the nose, nasopharynx or tonsils and the material thus collected was rubbed up in broth which was used for "shake" pour blood plates. Some of this broth suspension was also inoculated intraperitoneally into white mice. If these succumbed, pour blood plates

were made from the heart's blood. Material obtained by nasal irrigation with Ringer's solution was also used for pour blood plates. The blood plates were incubated for forty-eight hours and the colonies showing definite macroscopic zones of hemolysis were fished for further study. The strains were kept on blood-streaked agar and transferred fortnightly pending the comparative tests.

The data as to the individual case, the type of disease, the source of the culture, how obtained (from mouse or direct plate), and the number of examinations made on the patient are given in tables 1 to 6. The scheme used for numbering the cultures is exemplified by the following complex numbers, 112, II, 4. The first number refers to the case from which material was obtained for bacteriological examination. As hemolytic streptococci were frequently not obtained there is no sequence in these numbers. The second or Roman numeral refers to the different specimens taken at different intervals from the same patient. In the majority of cases only one specimen was taken. In one specimen from a case, hemolytic streptococci might be absent but present in a subsequent specimen, which accounts for the absence of some numbers. The last (Arabic) number refers to the strains isolated from the material. Omitted strain numbers are accounted for by loss of cultures or by failure to show sufficient hemolysis on subsequent tests to warrant inclusion in this series.

HEMOLYSIS TESTS

The first reaction studied was hemolytic activity. All of our strains were tested by both the plate and tube methods. The pour blood plates were observed according to the directions given by Brown (9). The changes in the blood plate are given according to his terminology. This reaction is discussed more fully in another paper in this series (10). The tube method employed was also that of Brown. This method was as follows:

To each of two tubes was added 1 cc. of an eighteen-hour broth culture diluted with saline 1-10 and 1-20 respectively. A drop of defibrinated horse blood was then added to each and the tubes were

incubated in the water bath for two hours at 37°C. The results were then read. This method was selected because it gives more quantitative information than the method adopted as a standard by the United States Army.

As noted above, the primary selection of streptococci as hemolytic was based on the presence of macroscopically visible clearing in the original pour blood plates. On replating the cultures and observing them after forty-eight hours incubation followed by forty-eight hours on ice, it was evident that we were dealing with three types of colonies as regards hemolysis (Brown). The colonies of the alpha type (definitely green and showing only slight if any clearing) were discarded. This type is considered in another article in this series (11). The remaining cultures fell in either the "alpha prime" or "beta" groups. The "alpha prime" type is characterized by colonies having a distinct zone of clearing, varying in diameter from 2 to 5 mm., at the end of forty-eight hour incubation. When examined under the microscope, the red blood cells are intact immediately around the colony, with the zone of clearing beyond either complete or partial. The "beta" type at the end of forty-eight hours incubation in pour blood plates shows a complete destruction of the red blood cells about the colony and in the area beyond, as determined by microscopic examination. In general the outer edge of the hemolytic zone is more clear cut than in the "alpha prime" types.

The test tube method has added no information. As is evident from the table there is no absolute correlation in the amount of hemolysis in the test tube and the reaction on the blood plate, except that the beta types as a group show a greater ability to hemolyze red blood cells in the test tube than the alpha prime types. The reaction in the blood plate, observed under the conditions advised by Brown, not only gives all the information that the tube method gives but also allows of a further separation into the two fairly distinct varieties (alpha prime and beta). The difference between the two types of colonies may not be very marked in some instances. Additional comparative work as reported in a paper in this series indicates

that the slight differences in hemolysis are due essentially to the action of two distinct hemolytic substances. If, as it seems, we are dealing with two different hemolytic substances, that is, with a difference in biologic action in the alpha prime and beta types we are then justified in utilizing this distinction as a primary basis for subdivision.

One group of cultures 168, V, 1, and W, I, 1, 2, 3 and 4 (table 3 and table 7) is distinctive.² With the blood plate method they give a beta type of hemolysis in that there is a complete hemolysis of the cells about the colony but the surrounding zone is comparatively narrow. With the test tube method these cultures give a slight hemolysis at most and frequently are negative.

Two factors probably enter into this apparent discrepancy. These cultures grow feebly in broth at best and it is probable that, at any one time, the accumulated amount of hemolysin is small because production does not outpace deterioration. In the blood plate, however, the hemolysin acts as it is formed and the resultant hemolysis on the plate is a truer index as to the nature of these strains. More detailed comparative data are given in another article in this series (10).

The above mentioned cultures, 168, V, 1 and W, I, 1, 2, 3, 4, showed an added peculiarity on the blood agar plate. Their colonies were characterized by a distinctive rosette shape. This characteristic remained constant on further cultivation. Such a colony would be classed by Brown as complex. However, he described no complex colonies of the beta type.

Another peculiar colony morphology was presented by a group of cultures of which serum strain 77 is the prototype. These colonies after twenty-four hours incubation were large, raised, mucoid and glistening; after forty-eight hours they had flattened and dried and when touched with the platinum wire appeared brittle. On further cultivation there was a marked tendency of these colonies to lose this peculiar characteristic. Nine cases yielded colonies which showed this colony morphology. The results of the tests on these strains are summarized in table 8.

² See Williams, Unneberg, Goldman and Hussey (11) for original description of these cultures under the terms "Witt" and "Witt like."

TABLE I

Pyogenes group

CASE NUMBERS	EXAMINATION NUMBER	STRAIN NUMBERS	TYPE OF DISEASE	TIME INCUBATED AT CULTURE	SOURCE	ISOLATED BY		HEMO- LYSIS	FERMENTATION OF					H ION	AGGLUTINATION SERUMS					SPONTANEOUS AGGLUTINATION
						Plate	Mouse		Blood plate	0.1 cc. 0.05 cc.	Lactose	Salicin	Mannite	Raffinose						
12	II	1	Tonsillitis	1 day	N.P.*	+	+	+	β^+	\times	+	+	+	+	++
12	III	1, 2	Rhinitis and	1 day	N.P.	+	+	+	β	\times	+	+	+	+	++
12	III	1, 2, 3, 6	Pharyngitis	1 day	N.P.	+	+	+	β	+	+	+	+	+	+
35	II	1, 2, 3	Normal	1 day	N.P.	+	+	+	β	+	+	+	+	+	+
81	I	3	Rhinitis	$\frac{1}{2}$ day	N.P.	+	+	+	β	+	+	+	+	+	+
117	I	3, 4, 5, 6	Influenza	1 day	N.P.	+	+	+	β	+	+	+	+	+	+
122	I	1, 2, 3, 4	Influenza	1 hr.	N.P.	+	+	+	β	+	+	+	+	+	+
141	I	1, 2, 3, 4	Influenza	1 day	N.P.	+	+	+	β	+	+	+	+	+	+
152	IV	1, 2, 5, 6			N.P.	+	+	+	β	+	+	+	+	+	+
152		7, 8			N.P.	+	+	+	β	+	+	+	+	+	+
171	II	1, 2, 3	Rhinitis	4 hrs.	N.P.	+	+	+	β	+	+	+	+	+	+
182	I	1, 2	Rhinitis	2 hrs.	N.P.	+	+	+	β	+	+	+	+	+	+
72	I	3	Rhinitis	2 hrs.	N.P.	+	+	+	β	+	\times	+	+	+	+
77	I	3, 4	Rhinitis and	$\frac{1}{2}$ day	N.P.	+	+	+	β	+	\times	+	+	+	+
77	I	2, 3, 4, 5	Pharyngitis		N.P.	+	+	+	β	+	\times	+	+	+	+
77	II	1	Normal		N.P.	+	+	+	β	+	\times	+	+	+	+
77	II	1, 2, 3, 4	Normal		N.P.	+	+	+	β	+	\times	+	+	+	+
77	II	5, 6			N.P.	+	+	+	β	+	\times	+	+	+	+

TABLE 2
Infrequens group

CASE NUMBERS	EXAMI- NATION NUM- BER	STRAIN NUMBERS	TYPE OF DISEASE	TIME ILL AT CULTURE	SOURCE	ISOLATED BY		HEMOLYSIS			FERMENTATION OF					H ION	AGGLUTINATION SERUMS			SPONTANEOUS AGGLU- TINATION
						Plate	Mouse	Blood plate	Tube method	Lactose	Salicin	Mannite	Raffinose	Saccharose	Dextrose broth		60	168 I, 1	77 I, 2	
168	I	1, 2	Influenza	1 day	N.P.	+	+	β	+	+	+	+	+	+	5.0	1,000	5,000	1,000	+	
168	II	1	Influenza	2 days	N.P.	+	+	β	+	+	+	+	+	+	4.9	...	3,000	...	+	
168	II	1, 2, 3, 4	Influenza	N.P.	N.P.	+	+	β	+	+	+	+	+	+	4.9	500	3,000	...	+	
168	IV	1, 2, 3	Influenza	5 days	N.P.	+	+	β	+	+	+	+	+	+	4.9	500	3,000	...	+	
168	V	2, 3	Influenza	8 days	N.P.	+	+	β	+	+	+	+	+	+	4.9	500	5,000	...	+	
18	I	4	Normal		N.P.	+	+	β	+	+	+	$\times 3$	± 3	+	5.1	—	—	...	+	
D.A.L.	—	60	Measles		Throat	+	+	β	+	+	+	+	+	+	5.0	10,000	200	1,000	—	

Dates of successive examinations: 168, I, on 2/10/20; 168, II on 2/11/20; 168, IV on 2/14/20; 168 V, on 2/19/20.

TABLE 3

Equi group

CASE NUMBERS	EXAMIN- ATION NUM- BER	STRAIN NUMBERS	TYPE OF DISEASE	TIME ILE AT CULTURE	SOURCE	ISOLATED BY		HEMOL- YSIS		FERMENTATION OF				H ION	AGGLUTINATION SERUMS								SPONTANEOUS AGGLUTINATION				
						Plate	Mouse	Blood plate	0.1 cc.	0.05 cc.	Tube method	Lactose	Salicin		Mannite	Raffinose	Saccharose	W 1, 3	23	3	84	32		273	60	168 I, 1	77 I, 2
112	I	1, 2, 4, 5	Pharyngitis and tonsil- litis	½ day	N.P.	+	+	β	+	+	+	+	+1	—	+	5.0	—	—	—	—	—	—	—	—	—	—	+
112	II	1, 2, 3, 4	Normal	—	N.P.	+	+	β	+	+	+	—	+1	—	—	5.0	—	—	—	—	—	—	—	—	—	—	+
116	I	1, 2, 3, 4	Rhinitis	¾ day	N.P.	+	+	β	+	+	+	—	+1	—	—	5.0	—	—	—	—	—	—	—	—	—	—	+
		5, 6, 7, 8	Rhinitis		N.P.	+	+	β	+	+	+	—	+1	—	—	5.0	—	—	—	—	—	—	—	—	—	—	+
167	I	1, 4, 6, 10	Influenza	4 days	N.P.	+	+	β	+	+	+	—	+1	—	—	4.9	—	—	—	—	—	—	—	—	—	—	+
168	V	1	Influenza	8 days	N.P.	+	+	β	—	—	—	—	+1	—	—	4.7	200	—	+
W	I	1, 2, 3, 4	Pleurisy	—	Pleura	+	+	β	×	—	—	—	+1	—	—	4.6	2000	—	+

Dates of successive examinations: 112 I, on 1/23/20; 112 II, on 3/5/20.

TABLE 4
Anginosus and salivarius (a) groups

CASE NUMBERS	EXAMINATION NUMBERS	STRAIN NUMBERS	TYPE OF DISEASE	TIME ELAPSED AT CULTURE	SOURCE	ISOLATED BY		HEMOLYSIS		FERMENTATION OF					H ION	AGGLUTINATION SERUMS						SPONTANEOUS AGGLUTINATION
						Plate	Mouse	Blood plate	0.1 cc. Tube method	0.05 cc.	Lactose	Saltin	Mannite	Raffinose	Saccharose							
13	I	1	Normal	—	N.P.	+		β	+	+	\pm 7	—	—	\pm 7	\pm 7	4.9	—	—	—	—	—	\pm
6	II	1, 2, 3, 4	Normal Pharyngitis Normal	— 2 hrs.	N.P. N.P. Tonsil N.P.	+		α'	\pm	\pm	\pm 2	—	—	\pm 1	\pm 1	4.9	—	—	—	—	—	\times
7	I	2				+		α'	\times	\times	\pm 1	—	—	\pm 1	\pm 1	5.3	—	—	—	—	—	\pm
32	I	4				+		α'	—	—	\times 1	—	—	\pm 3	\pm 1	5.3	—	—	—	—	—	\pm
40	II	1, 3				+		α'	\pm	\pm	\pm 1	—	—	\pm 1	\pm 1	5.3	—	—	—	—	—	\pm

TABLE 5
Salivarius (b) group

CASE NUMBERS	EXAMI- NATION NUM- BER	STRAIN NUMBERS	TYPE OF DISEASE	TIME ILL AT CULTURE	SOURCE	ISOLATED BY		HEMOLYSIS		FERMENTATION OF					H ION	AGGLUTINATION SERUMS						SPONTANEOUS AGGLUTINATION			
						Plate	Mouse	Blood plate	0.1 cc. 0.05 cc.	Lactose	Salicin	Mannite	Saccharose	23		3	84	32	273	60					
19	I	1	Rhinitis	5 days	N.P.	+		a	-	+	-	-	-	+	4.9	-	-	-	-	-	-	-	-	-	-
20	I	1, 2	Rhinitis	1 day	N.P.	+		a'	-	+	-	-	-	+	4.8	-	-	-	-	-	-	-	-	-	...
21	I	2	Rhinitis	1 day	N.P.	+		a'	-	+	-	-	-	+	5.0	-	-	-	-	-	-	-	-	-	+
32	I	5	Pharyngitis	2 hrs.	N.P.	+		a'	-	+	-	-	-	+	5.5	-	-	-	-	-	-	-	-	-	+
38	I	1, 2	Pharyngitis	$\frac{1}{2}$ day	N.P.	+		a'	-	+	-	-	-	+	5.0	-	-	-	-	-	-	-	-	-	+
81	I	2	Rhinitis	$\frac{1}{2}$ day	N.P.	+		a	-	+	-	-	-	+	4.9	-	-	-	-	-	-	-	-	-	...

TABLE 6

Mitis group

CASE NUMBERS	EXAMINATION NUMBER	SPECIMEN NUMBERS	TYPE OF DISEASE	TIME ELAPSED CULTURE	SOURCE	ISOLATED BY		HEMOLYSIS		FERMENTATION OF					MEDIUM	AGGLUTINATION SERUMS						SPONTANEOUS AGGLUTINATION			
						Plate	Mouse	Blood plate	0.1 cc. Tube method	Lactose	Salicin	Mannite	Raffinose	Saccharose		23	3	81	32	273	60		77 1, 2		
18	I	1, 2	Normal		N.P.	+		a'	++	++	++	++	++	++	5.4	—	—	—	—	—	—	—	—	—	+
18	I	5			Tonsil	+		a'	++	++	++	++	++	++	5.0	—	—	—	—	—	—	—	—	—	+
21	I	1	Rhinitis	1 day	N.P.	+		a'	—	++	×	—	—	—	5.0	—	—	—	—	—	—	—	—	—	+
21	I	3			N.P.	+		a	++	++	++	++	++	++	4.8	—	—	—	—	—	—	—	—	—	+
21	I	4			Tonsil	+		a'	++	++	++	++	++	++	5.2	—	—	—	—	—	—	—	—	—	+
32	I	2	Pharyngitis	2 hrs.	N.P.	+		a'	—	++	++	++	++	++	4.9	—	—	—	—	—	—	—	—	—	+
32	I	6			Tonsil	+		a'	++	++	++	++	++	++	4.8	—	—	—	—	—	—	—	—	—	+
33	I	1, 4, 5	Normal	—	N.P.	+		a'	++	++	++	++	++	++	5.0	—	—	—	—	—	—	—	—	—	+
	I	2, 6			N.P.	+		a'	++	++	++	++	++	++	4.9	—	—	—	—	—	—	—	—	—	+
72	I	2	Rhinitis	2 hrs.	N.P.	+		a'	++	++	++	++	++	++	4.8	—	—	—	—	—	—	—	—	—	+
113	I	2, 3,	Rhinitis	1 day	N.P.	+		a'	++	++	++	++	++	++	5.0	—	—	—	—	—	—	—	—	—	+
119	I	2, 3, 4, 6	Influenza	3 days	N.P.	+		a'	++	++	++	++	++	++	4.8	—	—	—	—	—	—	—	—	—	×
136	I	1, 2	Influenza	1 day	N.P.	+		a'	++	++	++	++	++	++	4.8	—	—	—	—	—	—	—	—	—	×

TABLE 7

Colony morphology "Like W"

CASE NUMBERS	EXAMINATION NUMBER	STRAIN NUMBERS	TYPE OF DISEASE	TIME ILL AT CULTURE	SOURCE	ISOLATED BY		HEMOLYSIS			FERMENTATION OF					H ION	AGGLUTINA- TION SERUMS		SPONTA- NEOUS AGGLUTI NATION
						Plate	Mouse	Blood plate	Tube method		Lactose	Salicin	Mannite	Raffinose	Saccharose				
									0.1 cc.	0.05 cc.									
168 W	V	1	Influenza	8 days	N.P.	+		β	—	—	—	—	—	—	pH	200	+		
	I	1, 2, 3, 4	Pleurisy	—	Pleura	+		β	×	—	—	—	—	—	4.7 4.6	2000	+		

TABLE 8
Colony morphology "Like 77"

CASE NUMBERS	EXAMINATION NUMBER	STRAIN NUMBERS	TYPE OF DISEASE	TIME ELAPSED AT CULTURE	SOURCE	ISOLATED BY		HEMOLYSIS		FERMENTATION OF					H ION	AGGLUTINATION SERUMS								SPONTANEOUS AGGLUTINATION
						Plate	Mouse	Blood plate	0.1 cc. 0.05 cc.	Lactose	Salicin	Mannite	Raffinose	Saccharose		23	3	84	32	273	60	77, 1, 2	168 1, 1	
167	I	1, 4, 6, 10	Influenza	4 days	N.P.	+	+	β	β	+	+1	-	-	+1	4.9	-	-	-	-	-	-	-	-	+
12	II	1	Tonsillitis	1 day	N.P.	+	+	β	β	+	+1	-	-	+1	5.0	+
12	III	1, 2, 3, 6	Rhinitis and pharyngitis	1 day	N.P.	+	+	β	β	+	+1	-	-	+1	5.0	1,000	-	5,000	5,000	-	-	-	-	+
12	III	1, 2	Pleurisy	-	Pleura	+	+	β	+	+	+1	-	-	+1	5.0	+
F			Pleurisy	-	Pleura	+	+	β	+	+	+3	-	-	+1	5.0	+
G			Pleurisy	-	Pleura	+	+	β	+	+	+3	-	-	+1	5.0	-	10,000	1,000	5,000	1,000	1,000	1,000	-	+
J			Pleurisy	-	Pleura	+	+	β	+	+	+7	-	-	+1	5.0	-	-	-	-	-	-	5,000	-	+
M			Pleurisy	-	Pleura	+	+	β	+	+	+1	-	-	+1	5.0	-	500	-	-	-	...	5,000	-	+
E			Pleurisy	-	Pleura	+	+	β	+	+	+7	-	-	+1	5.0	3,000	-	-	-	-	...	5,000	-	+
77	I	2	Rhinitis and pharyngitis	$\frac{1}{2}$ day	N.P.	+	+	β	+	$\times 3$	+1	-	-	+1	5.0	-	-	-	-	-	-	-	-	+
77	II	1	Normal	-	N.P.	+	+	β	+	$\times 3$	+1	-	-	+1	5.1	-	-	-	-	-	-	5,000	-	+
168	I	1	Influenza	1 day	N.P.	+	+	β	+	+1	+1	+1	-	+1	5.0	1,000	1,000	5,000	+

FERMENTATION TESTS

The next reaction considered was the fermentation of lactose, salicin, mannite, raffinose, saccharose. A highly satisfactory basic medium contained 0.5 per cent of agar neutral to the andrade indicator (used in a 1 per cent amount). To this medium was added 1 per cent of the test substance. This semi-solid medium was inoculated by means of a narrow pipette, containing a fresh eighteen hour broth culture. The pipette was thrust down into the medium and then withdrawn, 0.2 cc. being allowed to flow from the pipette during the withdrawal. The tubes were incubated at 37°C. and observations were made on the first, third and seventh days. The maximum degree of color change and the days on which the maximum color first appeared is recorded in the table.

In subdividing the strains on the basis of hemolysis and fermentative reactions, Brown's classification has been utilized as far as our data allowed. It will be noted that inulin was not employed. We omitted this carbohydrate because the hemolytic streptococcus ferments it so infrequently. This being the case the use of the Brown nomenclature seemed justified notwithstanding the omission of this test.

A moderate degree of variation has been noted in the fermentation tests. This variation has been limited to instances where the avidity for a fermentable substance was not great. That is, a slight positive result might on a successive test be negative or vice versa. In these instances the positive findings were recorded, as it was found that when a large number of tests were made on a given strain more positive results were obtained.

In general the fermentation reactions of the beta types were more constant and they showed a greater fermentative capacity, in that positive reactions were stronger and appeared more promptly. Occasional exceptions were encountered. The most noteworthy example is seen with all of the strains of case 77 and with strains 72 I, 3 (table 1). The slight reaction with lactose was uniformly elicited and in itself warrants the separation of these cultures from the other pyogenes varieties. Such a peculiarly low but constant avidity for a fermentable sub-

stance has apparently a definite classificatory significance among streptococci. As will be noted later these strains are serologically alike. We had encountered this peculiar low avidity for one sugar in cultures obtained during an investigation of an epidemic of septic sore throat. In that instance the epidemic strain was separable from other strains by a low avidity for salicin.

In table 9 the strains from the same case which showed differences in biological reaction are compiled. The instances where all the strains from the same case are alike in so far as studied, are easily seen in the preceding tables. Comparison shows how relatively few cases yielded dissimilar strains. This is probably no index as to number of varieties present in a single case because the number of strains obtained from each case was relatively few.

DETERMINATION OF FINAL HYDROGEN ION CONCENTRATION IN DEXTROSE BROTH

Avery, and Cullen (12) and Brown (13) have recorded differences between strains of human and of bovine origin in relation to the final hydrogen ion concentration in dextrose broth. The hydrogen ion determination was carried out with our series of cultures in the hope that some differences might be detected. The beef infusion broth was made up with 1 per cent dextrose, and had a reaction of pH 7.4 after sterilization. It was inoculated with 0.2 cc. of eighteen hour broth cultures. The colorimetric method was used and readings were made after forty-eight hours incubation. None of the human cultures gave as low a pH reading as most of the bovine controls. The group (W. 168, table 7) already mentioned as distinctive with regard to colony morphology and hemolysis, was characterized by giving the lowest pH value among the strains studied.

The number of groups possible among the streptococci on the basis of hemolysis and fermentative reactions, the relatively low case incidence of hemolytic streptococci in the inflammatory conditions and the fact that when present, hemolytic streptococci represent only a very small fraction of the flora, even in the early acute stages, indicates without immunological study that a dominant variety of hemolytic streptococcus was not present among the cases investigated.

TABLE 9

Isolations from individual cases showing differences in biological reactions

CASE NUMBERS	EXAMINA- TION NUMBER	STRAIN NUMBER	TYPE OF DISEASE	TIME IN AT CULTURE	SOURCE	ISOLATED BY		HEMOLYSIS		FERMENTATION OF					H ION	
						Plate	Mouse	Blood plate	Tube method		Lac- tose	Salicin	Man- nite	Rafi- nose		Saccha- rose
									0.1 cc.	0.05 cc.						
																pH
					N.P.	+		a'	-	+	×	+	-	+	+	5.0
21	I	1	Rhinitis	1 day		+		a'	+	+	+	+	-	+	+	5.0
21	I	2	Rhinitis	1 day		+		a	-	+	+	+	-	+	+	4.8
21	I	3	Rhinitis	1 day		+		a'	+	+	+	+	-	+	+	5.2
21	I	4	Rhinitis	1 day		+		a	+	+	+	+	-	+	+	4.9
32	I	2	Pharyngitis	2 hrs.		+		a'	+	+	+	+	-	+	+	5.3
32	I	3	Pharyngitis	2 hrs.		+		a	+	×	+	+	-	+	+	5.3
32	I	4	Pharyngitis	2 hrs.		+		a'	-	-	+	+	-	+	+	5.5
32	I	5	Pharyngitis	2 hrs.		+		a	-	-	+	+	-	+	+	4.8
32	I	6	Pharyngitis	2 hrs.		+		a'	+	+	+	+	-	+	+	4.8
72	I	2	Rhinitis	2 hrs.		+		β	+	+	×	+	-	+	+	5.1
72	I	3	Rhinitis	2 hrs.		+		a	+	+	+	+	-	+	+	4.9
81	I	2	Rhinitis	1 day		+		β	-	-	+	+	-	+	+	4.9
81	I	3	Rhinitis	1 day		+		β	+	+	+	+	-	+	+	5.0
168	I	1	Influenza	1 day		+		β	+	+	+	+	-	+	+	4.9
168	V	2	Influenza	8 days		+		β	+	+	+	+	-	+	+	4.9
168	V	1	Influenza	8 days		+		β	-	-	-	+	-	-	+	4.7

AGGLUTINATION TESTS

In the serological investigation of the cultures, the type strains of Dochez, Avery and Lancefield were used to prepare serums for preliminary study. This was done by inoculating sheep with increasing doses of killed cocci followed by injections of live cocci. Antiserums were also prepared by immunizing rabbits with selected cultures of our own series (see below). All injections were given intravenously.

For direct agglutination the technic employed was as follows:

The cultures were grown in 20 cc. of beef broth containing 1 per cent of peptone and of a final reaction after sterilization of pH 7.8. After incubation for eighteen hours the tubes were inspected and if the growth was sufficiently diffuse the upper portion was poured off, with care to avoid the sediment. This decanted portion was utilized as the antigen. If too heavy, dilution was made with broth. The serum was diluted with 0.85 per cent salt solution, and 0.1 cc. of serum dilution and 0.9 cc. of broth culture were placed in small test tubes. The mixtures were incubated at 55°C. for two hours and then read. If incubated longer or if held on ice spontaneous agglutination was found to interfere.

Some cultures when grown in broth were clear or nearly clear due to spontaneous agglutination. In some instances these cultures on thorough shaking, followed by a short period of sedimentation gave a suspension which could be used. With other self agglutinating strains occasional transplants grew without spontaneous agglutination and these could be utilized. However, with quite a number of strains the degree of spontaneous agglutination was so marked and constant that they could not be used at all for agglutination tests. In general these differences as to spontaneous agglutination occurred irregularly without regard to any determinable factors. Many expedients have been tried to prepare uniformly suitable suspensions but without success. The tabulated results, therefore, are the optimum results and give no indication of the repeated attempts and the difficulties encountered.

The results of the direct agglutinations with the serums prepared with the Dochez, Avery and Lancefield strains are given in tables 1 to 8. The primary interest is evidently with the pyogenes and infrequens groups to which the serum strains belong. In the pyogenes group there are many instances of moderate or marked agglutination by the serums. With some strains this was evident with two or more of the serums. With other strains the action was only marked with one of the serums. Serum 3 agglutinated more strains than any of the other serums.

ABSORPTION TESTS

The next step in the study was the absorption of the serums with the strains agglutinated and also with a certain number of the strains which gave such a marked degree of spontaneous agglutination that no determination of their serological relationship could be made by direct agglutination.

The technique of the absorption with these serums and also with the other serums mentioned below was as follows:

Broth cultures of the strains were centrifuged. The volume of packed cocci was read from the calibrations of the centrifuge tube and sufficient serum and salt solution were added to give a dilution of serum of 1 to 15, and keep the proportion of the volume of cocci to the total volumes of cocci, serum, and salt solution, as 1 is to 4. After thorough mixture, incubation in the water bath was carried out at 45°C. for three hours, the tube being shaken every fifteen minutes. The mixture was then placed on ice. The next morning the diluted serum was clarified by centrifuge and used for the test.

The unabsorbed serum was also used for comparison. The actual readings are given in the tables, not numerical values as were given in the tables of direct agglutination.

The results of the absorption of serums, D, A, and L, numbers 23, 3, 84, 32 and 273, are given in tables 10 to 14. It will be noted that in spite of considerable and often high cross-agglutination, only a very few absorption identities were encountered. The strains of case 122, I (influenza) absorbed the specific agglutinins of serum D, A, L, 32. Strain E (pleurisy) absorbed the agglutinins of serum D, A, L 23.

TABLE 10
Agglutinin absorption of serum D.A.L. 23

STRAIN NUMBER	TABLE NUM- BER	GROUP	AGGLUTINATION UNABSORBED SERUM							ABSORBED BY	AGGLUTINATION ABSORBED SERUM																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																														
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			500	1,000	2,000	5,000	10,000	20,000	Control		150	500	5,000	Control	150	500	5,000	Control																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							
12111, I.	1	Pyogenes	++	12111, I.	++	++	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

The table reads from left to right; thus strain B is followed by the symbols of the degree of reaction with serum D.A.L. 23. In the next column (absorbed by) the number is repeated to show that this strain was used for absorption and to indicate that the symbols immediately following, under the heading "absorbing strain," is the degree of agglutination by serum D.A.L. 23 after absorption by culture B. All the symbols under the heading "serum strain" refer to the agglutination of strain D.A.L. 23 by the serum after absorption by the strains as noted under the heading "absorbed by."

In this table and succeeding tables the last strain is homologous to the serum and the symbols under "absorbing strain" are duplications, as in these instances the serum strain is the same as the absorbing strain.
... Not readable because of spontaneous agglutination.

TABLE 11
Agglutinin absorption of serum D.A.L. 3

STRAIN NUMBER	TABLE NUM- BER	GROUP	AGGLUTINATION UNABSORBED SERUM							ABSORBED BY	AGGLUTINATION ABSORBED SERUM						
			500	1,000	2,000	5,000	10,000	20,000	Control		150	500	5,000	Control	150	500	5,000
12II, 1	1	Pyogenes	++	12II, 1	++	++	++	++
117I, 3	1	Pyogenes	++	117I, 3	++	++	++	++
141I, 1	1	Pyogenes	+	—	—	—	—	—	—	141I, 1	—	—	—	—	++	++	++
152IV, 1	1	Pyogenes	++	++	+1	+	+	+	+	152IV, 1	+	+	+	++	++	++	++
171II, 1	1	Pyogenes	++	++	++	++	++	++	+	171II, 1	+	+	+	++	++	++	++
G	1	Pyogenes	++	++	++	++	++	++	+	G	+	+	+	++	++	++	++
J	1	Pyogenes	++	J	++	++	++	++
B	1	Pyogenes	++	++	++	++	++	++	+	B	+	+	+	++	++	++	++
M	1	Pyogenes	++	++	+	+	+	+	+	M	+	+	+	++	++	++	++
C	1	Pyogenes	++	C	++	++	++	++
ZI	1	Pyogenes	+	—	—	—	—	—	—	ZI	—	—	—	—	++	++	++
D.A.L. 3	1	Pyogenes	++	++	++	++	++	++	—	D.A.L. 3	+	—	—	—	++	++	—

TABLE 12
Agglutinin absorption of serum D.A.L. 84

STRAIN NUMBER	TABLE NUM- BER	GROUP	AGGLUTINATION UNABSORBED SERUM							ABSORBED BY	AGGLUTINATION ABSORBED SERUM						
			500	1,000	2,000	5,000	10,000	20,000	Control		150	500	5,000	Control	150	500	5,000
12III, 1	1	Pyogenes	++	12III, 1	++	++	++	Control
G	1	Pyogenes	++	+1	+	+	+	+	+	G	+	+	+	+	+	+	+
B	1	Pyogenes	++	++	++	++	++	++	+	B	—	—	—	—	—	—	—
C	1	Pyogenes	++	C	+	+	+	+
77II, 1	1	Pyogenes	+1	+1	+1	+	+	+	+	77II, 1	+	+	+	+	+	+	+
D.A.L. 84	1	Pyogenes	++	++	++	++	++	++	—	D.A.L. 84	—	—	—	—	—	—	—

TABLE 13
Agglutinin absorption of serum D.A.L. 32

STRAIN NUMBER	TABLE NUM- BER	GROUP	AGGLUTINATION UNABSORBED SERUM							ABSORBED BY	AGGLUTINATION ABSORBED SERUM						
			AGGLUTINATION UNABSORBED SERUM								AGGLUTINATION ABSORBED SERUM						
			500	1,000	2,000	5,000	10,000	20,000	Control		150	500	5,000	Control	150	500	5,000
12III, 1	1	Pyogenes	++	12III, 1	++	++	++	Control
122I, 1	1	Pyogenes	++	++	++	++	++	+	-	122I, 1	+	-	-	-	-	-	-
122I, 2	1	Pyogenes	++	++	++	++	++	++	-	122I, 2	+	-	-	+	+	+	-
J	1	Pyogenes	++	J	+	+	+	-
B	1	Pyogenes	++	+	-	-	-	-	++	B	-	-	-	+	+	+	-
C	1	Pyogenes	++	C	+	+	+	-
D.A.L. 32	1	Pyogenes	++	++	++	++	++	+1	-	D.A.L. 32	+	-	-	+	+	+	-

TABLE 14
Agglutinin absorption of serum D.A.L. 273

STRAIN NUMBER	TABLE NUM- BER	GROUP	AGGLUTINATION UNABSORBED SERUM							ABSORBED BY	AGGLUTINATION ABSORBED SERUM				
			500	1,000	2,000	5,000	10,000	20,000	Control		150	500	5,000	Control	
17III, 1 G B C D.A.L. 273	1	Pyogenes	++	+1	+1	+	+	+	17III, 1	+	+	+	+	Control	
	1	Pyogenes	++	++	++	++	-	-	G	+	+	+	+	-	
	1	Pyogenes	++	++	++	++	-	-	B	+	+	+	+	-	
	1	Pyogenes	+	C	+	+	+	+	-	
	1	Pyogenes	++	++	++	++	+	+	D.A.L. 273	+	-	-	-	-	

Because of the peculiar colony characteristics of the strains from case 77, an anti-serum was prepared for this strain, rising rabbits. This serum on direct agglutination reacted with all the strains of case 77 and with some of the other strains which fall fermentatively into the pyogenes group. The absorption results, table 15, show that all the strains from case 77 are alike. The other strains acted upon by this serum have not the capacity of absorbing the specific agglutinins. One strain, case 72, I, 3 (the only one which showed the same peculiar fermentative reaction as 77) was also used for absorption, although no direct agglutination reaction could be carried out because of spontaneous agglutination. This strain absorbed the agglutinins from serum 77. We have no data on the colony morphology. If the colony had been characteristic at the time of isolation, this point was overlooked. When the peculiar fermentative reactions of the strain were observed there was nothing characteristic about its colonies but by this time the colonies of serum strain 77 had lost their original peculiarities. The reactions of serum 77 with strains having colony characteristics similar to serum strain 77 but falling in other than the pyogenes group are discussed later.

Strain 18, I (4) and the strains isolated from case 168 with the exception of 168 V 1, fell in the infrequens group and are given in table 2. Only slight reactions were observed with D. A. L. 60 serum. An absorption, carried out with strain 168, I, 1 which gave the highest reaction in this group, resulted negatively (table 16). A rabbit serum for strain 168, I, 1 was prepared because of the similarity of its colony morphology to that of strain 77. All of the strains of case 168, with one exception (strain 168, V, 1) are absorptively alike (table 17). The one exception also differed from the other strains in its fermentative characteristics.

Six cases yielded cultures which fell in the equi group fermentatively, in that they fermented salicin but failed to ferment lactose, mannite and raffinose. These strains can be divided into two subgroups. Strains from cases 112, 116 and 167 form one group. Strains from case W and strain 168 V, (1) differ

TABLE 15
Agglutinin absorption of serum 77 I, 2

STRAIN NUMBER	TABLE NUMBER	GROUP	AGGLUTINATION UNABSORBED SERUM							ABSORBED BY	AGGLUTINATION ABSORBED SERUM						
			AGGLUTINATION UNABSORBED SERUM								Absorbing strain		Serum strain				
			500	1,000	2,000	5,000	10,000	20,000	Control		150	500	5,000	Control	150	500	5,000
12II, 1	1 and 8	Pyogenes	12II, 1 G	+	+	+	+	+
	1 and 8	Pyogenes	+	+	+	+	+	+	+		+	+	+	+	+	+	+
J	1 and 8	Pyogenes	+	+	+	+1	+	+	+	J	+	+	+	+	+	+	+
M	1 and 8	Pyogenes	+	+	+	+1	-	-	-	M	-	-	-	+	+	+	+
168I, 1	2 and 8	Infrequens	+	+	-	-	-	-	-	168I, 1 72I, 3	-	-	-	+	+	+	+
	1	Pyogenes	+		+	+	+	+	+	+	+
77I, 2	1 and 8	Pyogenes	+	+	+	+	+	+	+	77I, 2	+	+	+	+	+
77I, 3	1 and 8	Pyogenes	+	+	+	+	+	+	+	77I, 3	+	+	+	+	+	+	+
77II, 1	1 and 8	Pyogenes	+	+	+	+	+	+	+	77II, 1	+	+	+	+	+	+	+
*77II, 1	1 and 8	Pyogenes	+	+	+	+	+	+	+	77II, 1	+	+	+	+	+	+	+

* Isolated from mouse.

TABLE 16
Agglutinin absorption of serum D.A.L. 60

STRAIN NUMBER	TABLE NUMBER	GROUP	AGGLUTINATION UNABSORBED SERUM							ABSORBED BY	AGGLUTINATION ABSORBED SERUM						
			AGGLUTINATION UNABSORBED SERUM								AGGLUTINATION ABSORBED SERUM						
			500	1,000	2,000	5,000	10,000	20,000	Control		150	500	1,000	2,000	5,000	10,000	20,000
168I, 1	2 and 8	Equi	+	+	+	+	+	+	168I, 1	+	+	+	+	+	+	+	
D.A.L. 60	2	Equi	+	+	+	+	+	+	D.A.L. 60	+	+	+	+	+	+	+	

TABLE 17
Agglutinin absorption of serum 168 I, 1

STRAIN NUMBER	* TABLE NUMBER	GROUP	AGGLUTINATION UNABSORBED SERUM							ABSORBED BY	AGGLUTINATION ABSORBED SERUM						
			AGGLUTINATION UNABSORBED SERUM								AGGLUTINATION ABSORBED SERUM						
			500	1,000	2,000	5,000	10,000	20,000	Control		Absorbing strain		Serum strain				
D.A.L. 60	2	Infrequens	—	—	—	—	—	—	D.A.L. 60	150	500	5,000	Control	150	500	5,000	Control
168II, 1	2	Infrequens	++	++	—	—	—	—	168II, 1	—	—	—	—	—	++	++	++
168V, 1	3 and 7	Equi	+	+	+	+	+	+	168V, 1	—	+	+	+	+	+	+	+
168V, 2	2	Infrequens	++	++	++	++	++	++	168V, 2	—	+	+	+	+	+	+	+
168I, 1	2 and 8	Infrequens	++	++	++	++	++	++	168I, 1	—	+	+	+	+	+	+	+

TABLE 18
Agglutinin absorption of serum W I, 3

STRAIN NUMBER	TABLE NUMBER	GROUP	AGGLUTINATION UNABSORBED SERUM								ABSORBED BY	AGGLUTINATION ABSORBED SERUM							
			AGGLUTINATION UNABSORBED SERUM									Absorbing strain				Serum strain			
			500	1,000	2,000	5,000	10,000	20,000	Control	150		500	5,000	Control	150	500	5,000	Control	
112I, 1	3	Equi	+	+	+	+	+	+	+	112I, 1	+	+	+	+	+	+	+		
116I, 1	3	Equi	+	+	+	+	+	+	+	116I, 1	+	+	+	+	+	+	+		
168V, 1	3 and 7	Equi	+	+	+	+	+	+	+	168V, 1	+	+	+	+	+	+	+		
WI, 1	3 and 7	Equi	++	++	++	++	++	++	++	WI, 1	+	+	+	+	+	+	+		
WI, 3	3 and 7	Equi	++	++	++	++	++	++	++	WI, 3	+	+	+	+	+	+	+		

from the first group in their hemolytic activity, in the final hydrogen ion concentration in dextrose broth and in their peculiar colony morphology. Because of these peculiarities an antiserum was prepared (rabbit) for strain W, I,₃ of the second group. This serum agglutinated equally well all the "W" strains, but gave only a slight reaction with strain 168, V, 1 and none with the strains from cases 112, 116, and 167. The cultures of the equi group were not agglutinated by the serums made from strains of the pyogenes group.

The absorption of serum W, 1, 3 (table 18) showed that the W strains were identical, but that strain 168, V, 1, was different from these. Absorption tests of serum, W, I, 3, with two strains 112 and 116 of the equi group were carried out with negative results. These strains served as negative controls. It is worthy of emphasis that the W strains from pleurisy, approach the bovine type of streptococcus in many of their characteristics.

The strains which fell into the three groups, *Anginosus*, *Salivarius* a and b, and *Mitis* are given in tables 4, 5 and 6 and consisted mainly of the alpha prime type. These groups are small and the majority of the cultures were not usable for agglutination tests, because of their extreme spontaneous agglutination. On account of this fact, and in the light of the failure to obtain similarities in the larger groups, the preparation of further serums did not seem indicated. The serums of other groups were tried with alpha prime strains but gave no reactions, which is of interest in comparison with the cross reactions where serum strain and strains tested belong in the same fermentative group.

COLONY PECULIARITIES

The colony distinctiveness of two groups of cultures "like 77" and "like W," have been mentioned previously in this paper. In the summary of the strains with the colony morphology "like 77" (table 8) it will be noted that the colony similarity is not paralleled by common fermentative characteristics. The direct agglutinations are given in the tables according to the

fermentative groups in which the strains belong. The results of the absorption tests (table 15) showed that none of the strains which, as far as observed, had this colony morphology were capable of absorbing specific agglutinins from serum 77. Strain 72, I, 3 included in table 1 on account of its cultural similarity to strain 77 but whose colony formation had not been studied at the time of isolation is seen to be absorptively identical with strain 77. As noted under the discussion of the fermentation tests, this strain was the only one encountered which was similar to the strains from case 77 in having peculiarly low avidity for lactose.

The other group having a colony peculiarity is represented by the W strains and strain 168 V, 1. These strains were alike and peculiarly distinct in all of the cultural and biological characteristics studied. Immunologically the W strains (isolated from the same specimen) are identical but strain 168, V, 1 differs from these (table 18). The results obtained with these distinctive groups of streptococci, show very clearly the fallacy of drawing conclusions as to the identity of strains even where the colony morphology and also all the other observed biological characteristics are alike. If then, in a series of cases of respiratory inflammations, there is found such a distinctive type, its presence is only suggestive as regards probable etiology. No conclusions can be drawn as to the identity of such strains until a serological investigation including agglutinin absorption has been made. Without such investigation, the emphasis placed by some investigators upon the frequent isolation from influenza cases of a streptococcus having distinctive cultural characteristics is wholly unjustified.

A further study of the strains isolated from the various cases noted did not seem indicated. As the organisms were studied the tendency was toward the separation of groups, the number of which increased in proportion to the number of characteristics studied, and the prospect of encountering a dominant type became less and less promising. Moreover, the possibility of further serological investigation was limited by the extreme spontaneous agglutination of some of the cultures. The

relatively low incidence of streptococcus hemolyticus and the relatively small numbers present when they did occur in inflammations of the mucous membranes also gave little indication that this organism was a common factor in any appreciable number of the cases investigated. Not only is there no evidence of a distinctive strain among the cases as a whole but likewise no indication of a distinctive strain common to any one of the types of disease studied.

CONCLUSIONS

A study of the streptococci of the hemolytic group obtained from inflammations or the respiratory tract shows that these strains fall into many cultural groups and sub-groups. A serological study showed so few similarities that the probability of a dominant strain seemed remote. There was no correlation between the grouping and the type of disease. These observations and the relative infrequency of streptococci of the hemolytic group in inflammations of the respiratory tract seem to justify the conclusion that none of the strains isolated were of primary etiological importance.

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STUDIES ON ACUTE RESPIRATORY INFECTIONS¹

IX. DIFFERENCES IN THE CHARACTER OF THE HEMOLYTIC ACTION OF STREPTOCOCCI AND THE RELATIVE VALUE OF VARIOUS METHODS IN DEMONSTRATING THESE DIFFERENCES

LUCY MISHULOW

From the Bureau of Laboratories, Department of Health, City of New York

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Differences in the hemolytic activity of streptococci when grown on blood agar were noted by Schottmuller (1) in 1903. The blood-agar plate was generally employed to determine the hemolytic ability of streptococci until Lyall (2) (1914) advised the use of the tube method as a means of differentiation. Later Smith and Brown (3), using the blood agar plate method, introduced a new nomenclature to indicate the reactions obtained. Brown (4) elaborated these findings and gave detailed descriptions of the characteristics to be observed and advocated a classification based upon such observations. He gave a very complete review of the whole subject.

The superiority of either the plate method or the tube method depends upon the answers to the following questions: (a) Does the plate method bring out all the differential characteristics not shown by the latter method, or is the reverse true? (b) Does each method bring out certain characteristics? (c) Is there with either method a greater correlation between the results elicited and any determinable differences in the substances producing hemolysis?

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The observations in this paper are offered in answer to these questions.

The streptococcus cultures employed were forty-two strains from the series reported by Valentine and Mishulow (5).

	<i>Strains</i>
Naso-pharynx, inflamed or "normal"	27
Pleurisy, purulent	4
Mastoiditis, blood culture	2
Tonsilitis	1
Serological Types of Dochez, Avery and Lancefield (6)	6
Brown (7) bovine and human "Types"	2

The strains were kept on blood streaked agar slants. Fresh transfers were made before each experiment.

The methods of demonstrating hemolysis and the conditions of observation were as follows:

Blood plates. Two loops of an eighteen to twenty hour broth culture were transferred to a tube of sterile broth which was shaken thoroughly and two loops of this dilution were inoculated into 1 cubic centimeter of citrated horse-blood. Ten cubic centimeters of liquid veal-agar were added and the mixture then poured into a Petri dish. By this procedure 10 to 25 colonies were obtained and the well separated colonies could be studied in detail. The plates were observed after twenty-four and forty-eight hours incubation at 37°C. They were then placed in the refrigerator for forty-eight hours and again observed.

The results recorded in table 1 are those noted after forty-eight hours in the incubator. With the cultures studied, forty-eight hours of refrigeration did not bring out any additional differential points.

Tube hemolysis. (Lyall method.) The red cells from fresh defibrinated horse-blood were washed three times in 0.85 per cent saline, and a 5 per cent suspension in saline was made. To 1 cc. of this suspension of red blood cells was added 0.5 cc. of an eighteen to twenty hour broth culture (beef infusion, peptone 1 per cent, NaCl 0.5 per cent, reaction pH 7.8 to 7.9), and after the tubes were shaken the mixtures were placed in a water-bath at 37°C. for two hours and were again shaken every half hour to prevent sedimentation of the blood cells. After this incubation, all tubes in which hemolysis was not

complete were rapidly centrifuged to facilitate observation of the degree of hemolysis.

Brown's modification of the tube method. An eighteen to twenty hour broth culture was diluted 1 to 10 and 1 to 20 with physiological salt solution. To 1 cc. of each dilution was added 1 drop of sterile defibrinated horse-blood and the mixtures were incubated in a water bath at 37°C. for two hours. Readings were made in the same manner as by Lyall's tube method. The proportion of red blood cells in different samples of defibrinated horse-blood varied considerably. To obtain uniformity in the amount of red cells used in the successive tests, the defibrinated blood was centrifuged and serum was added or removed until the volume of cells was to the volume of serum as 1 is to 2. The same broth cultures incubated at 37°C. for eighteen to twenty hours were used in making the hemolytic tests by the three methods described.

In tabulating the character of the hemolysis on the blood plates (table 1) Smith and Brown's and Brown's nomenclature is employed. The strains that gave clear colorless zones of hemolysis in forty-eight hours with microscopically complete destruction of red blood cells around each colony were designated *beta*. Those strains that produced hemolysis macroscopically similar to the above, but the colonies of which on microscopic examination were found surrounded by blood cells, were termed *alpha* prime. The strains which produced no definite hemolysis after forty-eight hours incubation, but definite although usually narrow zones of clearing after forty-eight hours refrigeration, were termed *alpha*. The last group usually showed green discoloration in and about the colonies.

The tabulation of the results with the plate method (table 1) shows that of the 42 strains 32 gave the *beta* type of hemolysis, 6 the *alpha* prime, and 4 the *alpha* type.

The question was raised in a few instances as to whether a strain should be classified as a *beta* or an *alpha* prime type. Most of these doubtful strains were finally considered *alpha* prime types because cells, although few in number and partly disintegrated, were still discernible about the colonies.

TABLE 1

Results with blood pour plates and tube methods compared; also pH values and classification

STRAIN	BLOOD PLATE METHOD						TUBE METHOD			H-ION CONCENTRATION AFTER 48 HOURS INCUBATION IN 1 PER CENT DEXTROSE BROTH pH 7.8	CLASSIFICATION ACCORDING TO BLOOD PLATES AND FERMENTATION REACTIONS (BROWN)
	48 hours incubation at 37°C.						Broth culture				
	Hemolysis				Green colorations	Type	20 hours incubation at 37°C.				
	Macroscopic		Microscopic				Lyall method	Brown method			
	Degree	Size of zone <i>mm.</i>	Of cells around colony	Of cells in clear zone				1-10 dilution	1-20 dilution		
10 X	+	1-2	+	+	-	β	+	-	-	4.6	<i>S. equi</i>
11 X	+	1-2	+	+	-	β	×	-	-	4.6	<i>S. equi</i>
12 X	+	1-2	+	+	-	β	±	-	-	4.7	<i>S. equi</i>
13 X	+	1-2	+	+	-	β	×	-	-	4.6	<i>S. equi</i>
16S _v (1)	+	1-2	+	+	-	β	±	-	-	4.7	<i>S. equi</i>
112 (1)	+	5-6	+	+	-	β	+	+	+	5.0	<i>S. equi</i>
112 _{II} (1)	+	4-5	+	+	-	β	+	+	+	5.0	<i>S. equi</i>
116(1)	+	2-3	+	+	-	β	+	+	+	5.0	<i>S. equi</i>
B cow 18	+	2-3	+	+	-	β	×	-	-	4.6	<i>S. anginosus</i>
13	+	6	+	+	-	β	+	+	+	4.9	<i>S. anginosus</i>
B ₂	+	4	+	+	-	β	+	±	±	5.0	<i>S. pyogenes</i>
M ₂	+	4	+	+	-	β	+	+	±	5.0	<i>S. pyogenes</i>
12 _{III} (1)	+	5	+	+	-	β	+	+	+	5.1	<i>S. pyogenes</i>

TABLE 1—Continued

STRAIN	BLOOD PLATE METHOD						TUBE METHOD			H-ION CONCENTRATION AFTER 48 HOURS INCUBATION IN 1 PER CENT DEXTROSE BROTH pH 7.8	CLASSIFICATION ACCORDING TO BLOOD PLATES AND FERMENTATION REACTIONS (BROWN)	
	48 hours incubation at 37°C.						Broth culture					
	Hemolysis				Green colorations	Type	20 hours incubation at 37°C.					
	Macroscopic		Microscopic				Lysall method	Brown method				
	Degree	Size of zone	Of cells around colony	Of cells in clear zone				1-10 dilution	1-20 dilution			
	mm.											
72(3)	{	+					+	+	+	5.1	S. pyogenes	
		+	4-5	+	+	-	β	+	±			±
77aw	{	+						+	+	+	5.0	S. pyogenes
		+	4-5	+	+	-	β	+	+	+		
77(2)	{	+						+	+	+	5.0	S. pyogenes
		+	4-5	+	+	-	β	+	+	+		
77(II1)	{	+						+	+	+	4.9	S. pyogenes
		+	4-5	+	+	-	β	+	+	+		
117(3)		+	3-4	+	+	-	β	+	+	±	5.0	S. pyogenes
141(1)	{	+	4	+	+	-	β	+	+	±	5.0	S. pyogenes
								+	+	±		
152 ₄ (1)		+	4	+	+	-	β	+	+	±	4.9	S. pyogenes
171 _{II} (1)		+	6	+	+	-	β	+	+	+	4.9	S. pyogenes
182(1)	{	+	3-5	+	±	-	β	+	+	+	4.8	S. pyogenes
								-	±	±		
Z I		+	5	+	+	-	β	+	+	+	5.0	S. pyogenes
Z III		+	5	+	+	-	β	+	+	+	4.9	S. pyogenes
JC 65	{	+	5-6	+	+	-	β	+	±	±	4.8	S. pyogenes
								-				
D.A.L. 3		+	5-6	+	+	-	β	+	+	±	4.9	S. pyogenes
D.A.L. 23		+	5	+	+	-	β	+	+	±	5.1	S. pyogenes
D.A.L. 32		+	5	+	+	-	β	+	+	+	5.0	S. pyogenes
D.A.L. 84		+	4-6	+	+	-	β	+	+	±	5.0	S. pyogenes
D.A.L. 273	{	+	2-3	+	+	-	β	+	+	+	5.0	S. pyogenes
								+	±	×		
D.A.L. 60		+	5-6	+	+	-	β	+	+	+	5.0	S. infrequens

TABLE 1—*Concluded*

STRAIN	BLOOD PLATE METHOD						TUBE METHOD			H-ION CONCENTRATION AFTER 48 HOURS INCUBATION IN 1 PER CENT DEXTROSE BROTH pH 7.8	CLASSIFICATION ACCORDING TO BLOOD PLATES AND FERMENTATION REACTIONS (BROWN)	
	48 hours incubation at 37°C.						Broth culture					
	Hemolysis				Green colorations	Type	20 hours incubation at 37°C.					
	Macroscopic		Microscopic				Lyall method	Brown method				
	Degree	Size of zone	Of cells around colony	Of cells in clear zone				1-10 dilution	1-20 dilution			
168 _{II} (1)	{	+	4	+	+	-	β	+	±	±	4.9	<i>S. infrequens</i>
VI ₂ (1)	{	+	$\frac{1}{2}$ -1	±	+	±	α^1	+	+	±	5.0	<i>S. salivarius</i> (1)
32(5)	{	+	5-6	-	±	-	α^1	+	±	±	5.5	<i>S. salivarius</i> (2)
40 ₂ (1)	{	+	1-2	-	+	-	α^1	+	±	±	5.3	<i>S. salivarius</i> (2)
18(1)	{	+	2-3	-	+	-	α^1	±	±	-	5.4	<i>S. mitis</i>
33(6)	{	+	1-2	-	+	±	α^1	+	×	-	4.9	<i>S. mitis</i>
136(2)	{	+	1	±	+	-	α^1	+	±	±	4.8	<i>S. mitis</i>
21(3)	{	-	-	-	±	±	α	-	±	-	4.8	<i>S. mitis</i>
11(1)	{	-	-	-	±	+	α	+	×	-	5.0	<i>S. salivarius</i> (1)
20(2)	{	-	-	-	-	+	α	-	-	-	4.7	<i>S. salivarius</i> (2)
6(2)	{	-	-	-	×	+	α	-	-	-	5.1	<i>S. ignavus</i>

+, complete hemolysis.

±, strong but not complete hemolysis.

×, trace.

×, faint trace.

Duplicate results are given to indicate the maximum and minimum reactions on repeated tests. D.A.L., Dochez, Avery and Lancefield.

Two strains, 168 II (1) and S2 (1), gave wide clearcut zones of hemolysis, without any sign of red cells about the colonies. In the outer zone, however, there were visible on microscopic examination a few partly disintegrated cells. These strains were classified as *beta* types.

Five strains (168 V (1), 10X, 11X, 12X and 13X) caused only slight hemolysis on the blood plate after twenty-four hours. After forty-eight hours there was a well defined zone of hemolysis and on microscopic examination no red cells were found about the colonies. Strain 168 V (1) was slower in its hemolytic activity than the other four strains mentioned. In retests this strain occasionally showed no hemolysis after twenty-four hours. All of the five strains because of the disappearance of the red cells about the colony were classed as *beta* types.

The results obtained with the tube methods, compared with each other and with the type of hemolysis on the blood agar plate (table 1), are summarized as follows:

Beta type 32; with Lyall method = 32 positive, 0 negative.

Alpha prime type 6; with Lyall method = 6 positive, 0 negative.

Alpha type 4; with Lyall method = 1 positive, 3 negative.

Beta type 32; with Brown method = 26 positive, 6 negative.

Alpha prime type 6; with Brown method = 4 positive, 2 negative.

Alpha type 4; with Brown method = 0 positive, 4 negative.

The six *beta* strains (10X, 11X, 12X, 13X, 168 V (1) and B cow 18) which failed to hemolyse by the Brown method gave variable amounts of hemolysis by the Lyall method when retested. As noted above, five of these cultures (human origin) were also slower in producing hemolysis when grown on blood agar. These strains of human origin were characterized by further peculiarities. As is noted in table 1, the pH figure after growth in 1 per cent dextrose broth is the lowest for this group of strains. This figure pH 4.6 or 4.7 is usually indicative of bovine origin (Avery and Cullen (8); Brown (7)). This is the figure given by strain B cow 18 (bovine origin).

These five human strains grew slowly and the amount of growth was never heavy. The failure to hemolyse regularly by the Lyall method may be due to the two factors; namely,

the small amount of hemolysin present at any one time due to scant growth and the progressive deterioration of hemolysin in fluid media. The amount produced minus the amount deteriorated might be so small that either no reaction resulted or at most a slight reaction. When this amount of hemolysin was reduced by dilution (Brown's method) no reaction resulted. The hemolysis on the plate although slow was always well defined after forty-eight hours. The more regular end results obtained with the plate may be due to the traces of hemolysin which acted directly upon the cells as fast as formed, thus eliminating to a large extent a loss of effect due to deterioration. If this explanation is valid, the plate method reveals the effect of all or nearly all the hemolysin formed, whereas the tube method reveals only the effect of that which has not deteriorated.

It appears, therefore, that for the demonstration of hemolysis by streptococci the blood plate method is more reliable than the test-tube methods because a certain percentage of feebly growing streptococci may fail to show hemolysis in the test-tube. The plate method is also more valuable for preliminary classification because it brings out differences in hemolysis that the tube methods fail to show. Since the completion of this work Clawson (9) has published similar conclusions with regard to the value of the blood plate. The Brown modification of the tube method has not been of value with the human strains reported in this paper. This method was used by Brown primarily for the differentiation of streptococci of bovine origin.

The failure of the tube method to differentiate between the *beta* and *alpha* types, which were differentiable by the plate method, raised the question as to whether the mechanism of hemolysis might not be different with these two varieties. If an essential difference could be demonstrated, this would be added evidence of the value of the plate method as a means of preliminary differentiation.

The following experiments were designed primarily to determine whether hemolytic action might be due to an extracellular substance in the one case and an intracellular substance in the other.

An eighteen to twenty hour broth culture was shaken by hand to distribute the cocci evenly. 1 cc. of washed 5 per cent horse-blood was added to 0.5 cc. of a sample of the broth culture (Lyall's method) and the mixture was incubated in a water bath at 37°C. for two hours, as a control. The remainder of the culture was rapidly centrifuged and the sedimented bacteria were suspended in distilled water for extraction. This suspension was made slightly more concentrated than the original broth culture and was divided into two parts. One sample (C) was placed in the ice-box and the other was shaken in a mechanical shaker for two and a half hours. A portion of the shaken sample (A) was tested immediately after shaking and the remainder (B) after further extraction in the icebox overnight. To obtain the extracts, all of the suspensions were centrifuged and to the clear extract was added $\frac{1}{10}$ its volume of 9.0 per cent sodium chloride to make it isotonic. The sedimented bacteria from each of the three extracts were resuspended in physiological saline and were also tested separately in portions A', B', C'. In each instance 1 cc. of a 5 per cent suspension of washed red blood cells (horse) was added to 0.5 cc. of the test substance (extract or suspension of cocci after extraction) to determine the hemolytic activity.

The results are given in table 2 and show that the extracts (refrigerator) of the *alpha* types had hemolytic action which was as strong as the original broth culture, or stronger. Extraction by shaking tended to destroy the hemolysin. Culture

D.A.L. no. 32 (*beta* type), however, showed strong hemolysis immediately after shaking, which decreased after refrigeration. This result as well as that of extraction by refrigeration without shaking indicates that the hemolysin of this strain is more labile with regard to time than that of the *alpha* types.

The hemolytic activity possessed by the suspensions of the extracted cocci was relatively the same as with the original broth culture. The question whether this hemolysis was due to preformed substances or to the products of growth during the test remains unanswered.

No special study was made of methemoglobin production but as far as observed there was a sharp difference depending on whether extracts or suspensions of cocci were used; the former gave negative and the latter positive results when tested before refrigeration.

TABLE 2
Hemolytic activity of extracts of streptococci

STRAIN	TYPE BROWN CLASSIFICATION	CONTROL BROTH CULTURE BEFORE SEDI- MENTA- TION	EXTRACTS MADE BY						SALINE SUSPENSION OF SEDIMENT AFTER REMOVAL OF EXTRACTS							
			Shaking two and one-half hours				Standing in refrig- erator overnight (C)		Shaken portion				Refrigera- tor portion			
			Tested same day (A)		Tested after refrigera- tion over- night (B)											
			Hemolysis	Methemoglobin	Hemolysis	Methemoglobin	Hemolysis	Methemoglobin	Hemolysis	Methemoglobin	A'	B'	C'			
D.A.L. 32	β	β	+	±	+	×	×	-	×	-	+	±	+	-	±	-
			+	×	×	-	-	-	-	-	±	-	-	×	-	-
12X	β	β	×	×	-	-	-	-	-	-	-	-	-	-	-	-
			×	-	-	-	-	-	-	-	-	-	-	-	-	-
32(5)	α^1	α^1	+	+	±	-	×	-	+	-	+	+	+	-	+	-
			×	±	-	-	-	-	×	-	-	-	-	-	+	-
6(2)	α	α	±	+	×	-	-	-	+	-	+	×	+	-	+	-
			-	±	-	-	-	-	-	-	-	-	-	-	-	-

The tests for hemolysis were made by Lyall's tube method. For explanation of symbols see table 1.

TABLE 3
Hemolysis tested on successive days of incubation at 37°C.

STRAIN	TYPE BROWN CLASSIFICATION	ENTIRE BROTH CULTURE					CLEAR BROTH AFTER CENTRIFUGATION				
		1 day	2 days	3 days	4 days	6 days	1 day	2 days	3 days	4 days	6 days
		Hemolysis	Methemoglobin	Hemolysis	Methemoglobin	Hemolysis	Hemolysis	Methemoglobin	Hemolysis	Methemoglobin	Hemolysis
		Methemoglobin	Hemolysis	Methemoglobin	Hemolysis	Methemoglobin	Methemoglobin	Hemolysis	Methemoglobin	Hemolysis	Methemoglobin
D.A.L. 32	β	+	±	+	×	×	+	±	-	-	×
12X	β	±	-	×	×	-	×	-	-	-	-
32(5)	α^1	+	±	+	±	+	+	+	+	×	-
6(2)	α	×	±	±	±	-	×	-	×	×	-

Hemolysis was tested by Lyall's tube method.

The question as to the persistence of the hemolytic activity of broth cultures of the various types was next considered. Broth cultures were made and the whole culture as well as the clear broth obtained by centrifugation were tested for hemolytic activity after incubation of the cultures for one, two, three, four, and six days respectively, using Lyall's tube method (table 3).

The *beta* and *alpha* types could not be differentiated by this method when the whole culture was used. With the supernatant fluid, however, there was a distinct difference. The *beta* types showed an average greater hemolytic activity after twenty-four hours incubation, which disappeared on further incubation. On the other hand the *alpha* types showed an increased hemolytic activity on longer incubation (see table 3). This seems to indicate that the hemolytic action of the *beta* type is essentially due to an extracellular product, labile in character; whereas that of the *alpha* types is due to the direct action of the cocci or of the products of their autolysis. The methemoglobin reactions were irregular, but it is noteworthy that they were consistently weak with the clarified broth.

The resistance to heat of the substances in cultures which cause hemolysis and methemoglobin production was also investigated (Lyall tube method). The results of these experiments are given in table 4.

There was a marked difference in the results obtained with the *beta* and the *alpha* types in regard to this point. With both *alpha* types there was a disappearance of hemolytic activity after heating at 55°C. for five minutes, while the *beta* hemolysin, resisted heating for five to ten minutes. These results apparently correlate and strengthen the deduction drawn from the previous experiments. Again, the indications are that the cultures of the *beta* type contain a preformed and at least partially free hemolysin which resists heating for five to ten minutes. The hemolytic activity of the *alpha* types is apparently due to direct action of the cocci, or of substances liberated by autolysis. The heating for five minutes had probably killed a large number of cocci or inhibited their activity. As previous experiments indicate, the autolytic products had probably not developed in

appreciable amounts during the eighteen hours incubation. The result of both these factors was an absence of hemolysis.

The results obtained in these studies probably explain to some extent the conflicting opinions of previous workers as to whether or not the hemolytic activity of streptococci is due primarily to an extracellular substance. The varying results obtained by different workers may depend on the types of streptococci studied and the methods used for investigation. De Kruif (10) in a

TABLE 4

The resistance to heat of substances which cause hemolysis and methemoglobin production

STRAIN	TYPE BROWN	PERIOD OF HEATING AT 56°C.									
		0 minutes		5 minutes		10 minutes		15 minutes		20 minutes	
		Hemolysis	Methemoglobin	Hemolysis	Methemoglobin	Hemolysis	Methemoglobin	Hemolysis	Methemoglobin	Hemolysis	Methemoglobin
D.A.L. 32	β	+	\pm	+	—	×	—	×	?	—	—
81(3)	β	+	\pm	\pm	—	×	—	—	—	—	—
12X	β	\pm	—	\pm	—	—	—	—	—	—	—
32(5)	α^1	+	+	—	\pm	—	—	—	—	—	—
32(6)	α	×	—	—	—	—	—	—	—	—	—
20(2)	α	—	+	—	×	—	—	—	—	—	—
21(3)	α	—	+	—	—	—	—	—	—	—	—
6(2)	α	—	\pm	—	×	—	—	—	—	—	—

Lyall's tube method was employed in testing for hemolysis.

An eighteen hour broth culture was used in these tests.

review of work of this nature has shown very clearly that with his cultures hemolysin contained in the clear broth reached a maximum concentration and then rather rapidly disappeared. The whole culture, however, retained its hemolytic capacity for a much longer time than the clear broth. These results correspond with the results of the filtration experiments of Meader and Robinson (11). They found that when a broth culture was rapidly filtered there was considerable hemolysin in the filtrate; whereas with slow filtration the filtrate gave negative results.

The conclusion that the *alpha* types owe their hemolytic activity primarily to direct action of the cocci brings up the question as to the mechanism of this action. With both the *beta* and the *alpha* types there are other factors involved besides the mere liberation of the hemoglobin. This is plainly evident with the blood plate method, where hemolysis, is accompanied by the development of a clear transparent decolorized area. The action of bacteria upon the stroma and hemoglobin together with the mechanism of this action has been investigated by several workers. We have not considered this aspect of the problem but have limited our efforts to the demonstration of primary differences in hemolytic action that would correlate with the blood plate picture, which depends not only upon simple hemolysis but upon the activity of other factors as well.

CONCLUSIONS

The blood agar plate, incubated for forty-eight hours, is a more reliable method of determining the hemolytic action of streptococci because differences in hemolytic action can be observed with the blood agar plate which are not observable with the tube method.

The differences in hemolysis observed in the plate are correlated with primary differences in the mechanism of hemolysis. The hemolytic action of the *beta* type is primarily due to a hemolysin which is a soluble extracellular product. The hemolytic activity of the *alpha* types seems to be due to the direct action of the cocci or to products liberated by autolysis. The plate method, which brings out these points, is, therefore, the more valid method on which to base a classification.

A modification of the tube method, in which only the clarified broth is used for the test, appears to be an improvement on the regular method. However, it may fail in some instances to reveal hemolytic action where the plate method would give positive results.

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STUDIES ON ACUTE RESPIRATORY INFECTIONS¹

X. THE NATURE AND VALUE OF A SO-CALLED PRECIPITIN REACTION AS APPLIED TO THE SEROLOGICAL GROUPING OF STREPTOCOCCI

CHARLES KRUMWIEDE AND EUGENIA VALENTINE

From the Bureau of Laboratories, Department of Health, New York City

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Barnes, (1), in a study on the cultural and serological relationship of hemolytic streptococci reported recently that the serums of rabbits intensively immunized against the streptococcus will give precipitin reactions with streptococcus antigens even when such serums are diluted as high as 1-3200.

His method of eliciting what he terms a precipitin reaction, was as follows: The streptococci were grown in broth for forty-eight hours at 37°C. The broth cultures were centrifuged at high speed to sediment the cocci. In each test a constant amount (1 cc.) of clear undiluted supernatant broth antigen was added to an equal amount of the diluted antistreptococcus serum. These mixtures as well as controls with normal rabbit serum and with uninoculated broth were incubated at 37°C. for eight to ten hours, removed to the ice-box for two hours and then read. Sterility tests were made to eliminate the possibility of the precipitate being bacterial growth.

To us the results as reported by Barnes seemed most extraordinary. In our experience the most potent serums would when added to concentrated antigens, give precipitin reactions only in dilutions as high as 1:100 or 1:200 at most. Even the

¹ One of a series of studies carried out under the direction of William H. Park, Anna W. Williams and Charles Krumwiede. The previous studies in this series were reported in this Journal, 1921, vol. 6, no. 1. This investigation was made possible by a grant of money from the Metropolitan Life Insurance Influenza Fund for a part of the expense.

prolonged incubation period used by Barnes did not seem to us to explain his positive results with thousand-fold and greater dilutions of serum.

It seemed advisable, therefore, to investigate the reaction obtained by Barnes to determine whether his interpretation of its nature was correct. If the reaction were actually a precipitin reaction the relative specificity of the results would be open to question; in addition the applicability of the antibody-absorption method would be limited, because the absorption of precipitins gives, not infrequently, non-specific results (2). Even if Barnes' interpretation were not correct, the reaction might have an advantage over the regular agglutination method, by eliminating the all too frequently disturbing factor of spontaneous agglutination.

Our experiences with the method described by Barnes when controlled by the regular agglutination methods are not given here in full. The table sufficiently illustrates our findings. These results also suffice to show the nature of the reaction.

The striking point in the table is the difference in the reactions obtained with the method described by Barnes when parallel tests were incubated at 37°C. and at 55°C. respectively. At 55°C. there is seen a reduction of the degree of reaction within the limits to be expected with the precipitin reaction and which we actually obtained with culture filtrate antigens. If the reaction described by Barnes were a true precipitin reaction there should not be this difference at the two temperatures employed.

Numerous tubes in both series were used for bacteriological tests. The contents of each tube was added to a separate tube of blood agar and the mixtures poured into petri dishes. After incubation, abundant growth was obtained from all the samples of the 37°C. series but from none of the 55°C. series. Likewise, smear and hanging drop examinations of the sediment of centrifuged tubes of similar series incubated at 37°C., showed the presence of numerous streptococci. Where clumps were visible to the naked eye, clumps of streptococci were also seen in the hanging drop preparations. The constant evidences of growth

even from the saline control in every series incubated at 37°C. and the absence of such growth in the 55°C. series, absolutely contradicts Barnes' claim that the precipitate is not due to bacterial growth.

After we had obtained the above results, two statements in Barnes' article seemed very significant. He found that the addition of cresol 0.3 per cent "seriously interfered with the precipitin reaction." Likewise, he found that the supernatant broth from a culture gave "better results" than an extract from killed cocci. How much reaction was obtained and in what dilution, is not stated in regard to these extracts.

There is only one possible conclusion as to the mechanism of the reaction described by Barnes. Although with the immune serums there is some true precipitin action in the lower dilutions, the essential phenomenon is that of growth and agglutination of the cocci.

As to the value of the reaction it is evident from a study of the table that with cultures showing little tendency to spontaneous agglutination, the results were about the same as those obtained with the ordinary agglutination method. With cultures showing a marked tendency to spontaneous agglutination, no better results were obtained with the miscalled precipitin method which for convenience we shall term the growth-agglutination method. To determine the degree of spontaneous agglutination a comparison, dilution by dilution, of the results obtained with the immune and with the normal serum must be made. In the lowest dilutions the serum present may limit spontaneous agglutination because in these dilutions the cocci are growing in a medium qualitatively like serum broth. In the highest dilutions the amount of growth decreases and consequently the evidences of spontaneous agglutination become less marked.

In a few instances given in the table, the results have been somewhat better with the growth agglutination method than with ordinary agglutination. Thus with serum 3 and strain 3 the contrast between normal and immune serum is more marked. With the same serum and strains 23 and 171, the degree of group agglutination is less marked.

TABLE 1

SÉRUM*	STRAIN	AGGLUTINATION												REACTION, SERUM AND SUPERNATANT BROTH											
		Normal serum †						Immune serum						Normal serum †						Immune serum					
		37°C.						55°C.						37°C.						55°C.					
		10	100	500	1,000	5,000	C.	10	100	500	1,000	5,000	C.	10	100	500	1,000	5,000	10,000	20,000	C.	10	50	100	500
S. 60....	60 X	—	—	—	—	—	—	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+	+	—	—
	12 X	—	—	—	—	—	—	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+	+	—	—
R. 77....	77	—	—	—	—	—	—	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+	+	—	—
	25 X	—	—	—	—	—	—	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+	+	—	—
	32	—	—	—	—	—	—	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+	+	—	—
	3	—	—	—	—	—	—	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+	+	—	—
	1 X	—	—	—	—	—	—	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+	+	—	—
S. 3.....	3	+	+	+	+	+	—	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+	+	—	—
	111	+	+	+	+	+	—	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+	+	—	—
	23	+	+	+	+	+	—	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+	+	—	—
	171	+	+	+	+	+	—	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+	+	—	—
S. 23.....	23	—	—	—	—	—	—	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+	+	—	—
	3	+	+	+	+	+	—	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+	+	—	—
	23 X	+	+	+	+	+	—	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+	+	—	—
	12	+	+	+	+	+	—	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+	+	—	—
	122	+	+	+	+	+	—	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+	+	—	—

Symbols:—+, maximum reaction observed.

+1, +, +, X, decreasing degrees of reaction.

— = no reaction.

* Serums:—S. = sheep, R. = rabbit.

† Normal serum, serum of same species of animal as used for immunization.

These results indicate that the application of the method might be attempted if the possibility of obtaining results was sufficiently important to overbalance its drawbacks namely—the frequent failures due to spontaneous agglutination, the time for centrifuging, the excessive care necessary to prevent contamination and the difficulty of reading the relatively less marked reactions obtained. If this method were employed, the question would be raised immediately as to the applicability of the absorption method to evaluate the reactions obtained.

It does not seem of value to tabulate our absorption tests. As was to be expected from the data given in table 1 the results obtained were practically the same as with the regular agglutinin absorption method. Where spontaneous agglutination interfered with one method it interfered with the other. Somewhat greater difficulty was encountered with the growth method, as in some instances a larger absorbing dose was necessary than was the case with the regular agglutinin absorption method. In carrying out these absorptions the mixtures of serum and absorbing cocci were incubated at 55°C. to prevent growth of the absorbing strain in the subsequent tests. This method was satisfactory as was shown by the parallel tests with the regular agglutination method.

CONCLUSION

The method described by Barnes is not, as he concluded, a precipitin reaction. The reaction is due to the growth and agglutination of the cocci in the mixtures of broth and diluted serum. The results obtained by this method are essentially the same as those obtained by the ordinary agglutination method.

The method is of no especial value as regards the elimination of the factor of spontaneous agglutination which renders so difficult the serological investigation of streptococci.

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OBSERVATIONS UPON THE CONGLUTINATION PHENOMENON¹

FRANK MALTANER AND ELIZABETH JOHNSTON

*From the Division of Laboratories and Research, New York State Department of
Health, Albany*

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In a previous communication (1) it was shown that the agglutination of sheep red blood cells by calf serum was the result of the coagulation of fibrinogen contained in the serum. This coagulation was induced by the reaction between cytozyme present in the sheep cell suspension and the other elements necessary for clot formation supplied by the calf serum. The addition of blood platelets to fresh calf serum produced secondary clots and the fibrinogen-free serum thus obtained no longer agglutinated sheep cells. Calf sera also which possessed hemolytic action for sheep cells no longer reacted after removal of fibrinogen in this manner.

In 1902 (2) Ehrlich and Sachs recorded experiments which according to their interpretation demonstrated the union of complement with hemolytic amboceptor previous to the union of the latter with the red blood cells. In these experiments a mixture of heated bovine serum and fresh horse serum strongly hemolysed guinea-pig red blood cells, while separately the sera reacted slightly or not at all. Guinea-pig cells, however, which had been previously mixed with heated bovine serum and separated from the latter were not hemolysed by fresh horse serum, while heated bovine serum which had been removed from these cells previous to the addition of horse serum still retained its property of hemolysing other guinea-pig cells in the presence of fresh horse serum. They concluded that amboceptor was present in the heated bovine serum and that it could not unite with the blood cells until after its affinity for complement present in the fresh horse

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serum was satisfied. Bordet and Gay in 1906 (3) showed that the hemolysis and also the agglutination, which precedes the hemolysis and was not mentioned by Ehrlich and Sachs, were caused by a property peculiar to bovine serum which increased greatly both the agglutination and hemolysis of sensitized cells and required for its action fresh serum. In his own interpretation of the experiments of Ehrlich and Sachs, Bordet assumed the presence in bovine serum of a heat-stable substance (bovine colloid) which altered the molecular attraction in the mixture in such a manner as to increase the facility with which the cells were agglutinated and hemolysed by the normal antibodies and complement present. Bordet and Streng (4) later spoke of this reaction as "Conglutination" and showed that the sera of several other animals in addition to bovines gave rise to this phenomenon. They also showed that complement other than fresh horse serum could be used and that the blood cells of different animals and also various bacteria could be substituted for guinea-pig cells.

Our observations upon the rôle of fibrinogen in the agglutination and hemolysis of sheep cells by calf serum led us to think that the "bovine colloid" of Bordet might be identified as fibrinogen and that the relation of conglutination to the phenomenon of blood coagulation could be thus established. For this purpose fresh bovine and fresh horse serum were obtained and washed blood platelets were added as in our experiments with calf sera. Secondary clots were formed in both sera demonstrating the presence of fibrinogen, but a single re clotting in this manner did not always remove all of the fibrinogen. This was especially true of bovine serum, which could usually be re clotted three or four times by the addition of more platelets to the serum obtained from the successive clots. After no more clotting could be induced in either serum in this manner they were tested for their agglutinative and hemolytic action upon washed guinea-pig blood cells. No agglutination or hemolysis was observed in these tests. In control tests with the original untreated sera both agglutination and hemolysis of the guinea-pig cells were obtained.

Experiment 1. This experiment demonstrates the effect of the removal of fibrinogen from bovine and horse sera upon their agglutinative and hemolytic action for guinea-pig blood cells.

The experiment of Ehrlich and Sachs was then duplicated with the use of the fibrinogen-free sera and for purposes of control the original untreated bovine and horse sera. The bovine serum in each test was heated to 56°C. for one-half hour. In the tests in which fibrinogen-free sera were used no agglutination nor hemoly-

TABLE 1

FRESH SERUM 0.1 cc.	5 PER CENT SUSPENSION WASHED GUINEA-PIG CELLS 0.2 cc.	
	Agglutination	Hemolysis
Untreated horse serum.....	Partial	Partial
Untreated bovine serum.....	Strong	Complete
Fibrinogen-free horse serum.....	None	None
Fibrinogen-free bovine serum.....	None	None

The tests were incubated in the water-bath at 37°C. and reactions were recorded at the end of one hour.

TABLE 2

SERUM 0.5 cc.	5 PER CENT SUSPENSION WASHED GUINEA-PIG CELLS 1.0 cc.	
	Agglutination	Hemolysis
Fresh horse serum.....	Partial	Partial
Heated bovine serum.....	None	None
Mixture equal parts fresh horse serum and heated bovine serum.....	Strong	Complete
Fresh fibrinogen-free horse serum.....	None	None
Heated fibrinogen-free bovine serum.....	None	None
Mixture equal parts fresh fibrinogen-free horse serum and heated fibrinogen-free bovine serum.....	None	None

Tests were incubated in the water-bath at 37°C. and reactions were recorded at the end of one hour.

sis was obtained either with each serum alone or with a mixture of the two; while in the control test some agglutination and partial hemolysis were obtained with the fresh horse serum, no reaction was obtained with the heated bovine serum, and strong agglutination followed by rapid and complete hemolysis was produced with a mixture of the two.

Experiment 2. This experiment demonstrates the effect of the removal of fibrinogen from bovine and horse sera upon their "conglutinating" action for guinea-pig blood cells.

In order to determine whether the fibrinogen-free sera still possessed the heat-sensitive constituents necessary for the production of agglutination and hemolysis in the presence of fibrinogen, mixtures of these sera were made with each of the two original sera after inactivation of the latter at 56°C. for one-half hour. These mixtures were incubated with guinea-pig blood cells and agglutination and hemolysis were obtained with all of the mixtures.

TABLE 3

SERUM MIXTURE EQUAL PARTS 0.2 cc.	5 PER CENT SUSPENSION WASHED GUINEA-PIG CELLS 0.2 cc.	
	Agglutination	Hemolysis
Fresh fibrinogen-free horse serum and heated horse serum, containing fibrinogen.....	Strong	Partial
Fresh fibrinogen-free horse serum and heated bovine serum, containing fibrinogen.....	Partial	Strong
Fresh fibrinogen-free bovine serum and heated bovine serum containing fibrinogen.....	Strong	Complete
Fresh fibrinogen-free bovine serum and heated horse serum containing fibrinogen	Partial	Partial

Tests were incubated in the water-bath at 37°C. and reactions were recorded at the end of one hour.

Experiment 3. This experiment demonstrates the presence in fibrinogen-free bovine and horse sera of the heat-sensitive constituents necessary for the production of agglutination and hemolysis in the presence of fibrinogen.

Finally, in order to show that the agglutination and hemolysis by these two sera depended upon the presence of both fibrinogen and the heat-sensitive serum constituents, mixtures of fibrinogen-free horse and bovine serum and of the original two sera that had been inactivated at 56°C. for one-half hour were made and tested for their activity against guinea-pig cells.

No agglutination nor hemolysis was obtained with the mixture of heated sera, and no hemolysis but a slight agglutination with

the mixture of the two defibrinated but unheated sera which only became evident after an incubation of one hour at 37°C. This was probably due to the presence in one or both of the defibrinated sera of a little fibrinogen which was not removed during the technic of defibrination and which was coagulated by the excess of heat-sensitive constituents brought together in the mixture.

TABLE 4

SERUM MIXTURE EQUAL PARTS 0.2 CC.	5 PER CENT SUSPENSION WASHED GUINEA-PIG CELLS 0.2 CC.	
	Agglutination	Hemolysis
Fresh fibrinogen-free horse serum and fresh fibrinogen-free bovine serum.....	Slight	None
Heated horse serum, containing fibrinogen and heated bovine serum, containing fibrinogen....	None	None

Tests were incubated in the water-bath at 37°C. and reactions were recorded at the end of one hour.

Experiment 4. This experiment demonstrates that the agglutination and hemolysis of guinea-pig blood cells by mixtures of bovine and horse sera depend upon the presence of both fibrinogen and heat-sensitive constituents of the serum.

These experiments certainly show a close relation of the agglutinating and hemolytic properties of horse serum and bovine serum, both alone and in mixtures, with the elements responsible for the phenomenon of coagulation. The presence of fibrinogen together with heat-sensitive constituents of serum is shown to be essential for the production of agglutination, hemolysis and the phenomenon of conglutination as observed with these sera and guinea-pig blood cells. The close association of these phenomena becomes especially apparent in the application of the technic for re clotting the sera. In order to remove all of the residual fibrinogen from bovine serum it was usually found necessary to add several successive portions of platelets. As long as a clot could still be formed in the serum by these means some agglutination and hemolysis could also be obtained, although in a reduced degree.

SUMMARY AND CONCLUSIONS

1. The conglutination phenomenon described by Bordet was reproduced using a mixture of inactivated bovine serum and fresh horse serum with washed guinea-pig cells.

2. When the bovine and horse sera used were first depleted of their fibrinogen by secondary coagulation induced by treatment of the active sera with blood platelets, they no longer gave rise to this "conglutination" phenomenon.

3. Not only the conglutination of guinea-pig blood cells, but also the hemolysis and agglutination of these cells by both sera when used separately or in mixtures, depended upon the presence of fibrinogen and a heat-sensitive serum constituent.

4. The degree of agglutination or hemolysis obtained in the presence of the heat-sensitive serum constituents depends, in part at least, upon the quantity of fibrinogen present.

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COMPLEMENTING ACTIVITY OF THE BLOOD SERUM WITH RELATION TO ADRENAL DEFICIENCY.

ENRIQUE E. ECKER AND J. M. ROGOFF

From the Department of Pathology and the H. K. Cushing Laboratory of Experimental Medicine, Western Reserve University, Cleveland, Ohio

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Increasing attention is at present being given to the study of the direct or indirect relation of the endocrine organs to immune reactions. A number of studies have been limited to experiments on the effect of various toxic substances on the adrenal tissue, and direct *in vitro* experiments have been reported demonstrating a possible detoxifying property of adrenal products. Animal experiments have been limited by technical difficulties, and have sometimes been done with partial extirpation of the glands, leaving sufficient tissue to sustain life. Unsuitable animals have often been employed, frequently at times when the animals were practically moribund.

Josué and Paillard injected adrenalin and adrenal extracts into rabbits with no effect on the opsonic properties of the blood. Hektoen and Curtis found that adrenalectomy in normal dogs, and in dogs at the height of the antibody curve following injection of rat blood, did not cause any fall in the antibody content of the blood serum, as determined by hourly observations after the operation and until death. More recently Gates found that following the removal of three-quarters to seven-eighths of the adrenal tissue of guinea pigs and immunizing the animals to *B. typhosus* or to hen corpuscles, a comparison of the antibody curves with curves from control animals similarly treated fails to show that adrenalectomy had any influence upon the rise or persistence of the antibodies in the blood. This author claims that it does not seem probable that the small remainder

of adrenal tissue left *in situ* to sustain life would affect quantitatively the antibody response to a given antigen injection as do the entire normal glands. Gates found that if too much tissue were taken the animal showed characteristic symptoms and died at periods from a few hours to several days. Surviving guinea pigs recovered quickly from the operation and remained well for months, sometimes losing at first 50 to 100 grams in weight, which was often recovered later. These observations are confirmed by our own experiences with guinea-pigs.

Pinner points out in a recent report that injection of adrenalin and atropin into guinea-pigs lead to a decrease of complement. The evidence given above is that the adrenal glands play no important part in the production of antibodies and it is mainly in view of the report of Pinner that a study of the possible function of these glands or their products has been made to ascertain whether or not the complete removal of these organs in rabbits alters the complementary activity of their blood.

In our experiments the rabbit has been chosen as the experimental animal because of the fact that a fair proportion of these animals survive complete bilateral adrenalectomy. It has been assumed that the comparatively large number of survivals after double adrenalectomy in rabbits might be accounted for by the presence of accessory adrenal tissue. Accessory glands when present in the rabbit are usually situated along the cava or near the vessels of the kidney and can generally be detected without difficulty and removed during the operation. Such accessory adrenals consist only of cortical substance. Chemical and microscopic examinations fail to reveal the presence of any chromaffin material in them. At any rate, when accessory adrenal tissue is present in an animal the amount is much less than the fraction of adrenal which is generally found necessary to leave behind to sustain life.

Adrenalectomy was performed on five rabbits. In one rabbit both adrenals were excised, through an abdominal (median) incision, in a single operation; in the others the right adrenal was removed first and twelve days thereafter the left gland was excised. The glands were reached sometimes through the

abdomen, sometimes through an incision parallel with and just beneath the ribs, and more often through an incision in the lumbar region, reaching the gland without opening the peritoneum. A sixth rabbit was operated upon to serve as a control. In this animal, the abdomen was opened and the capsule of the left kidney split and peeled back. A seventh, unoperated rabbit was bled at the same intervals as the experimental animals to control possible variations from normal that may be due to the repeated small hemorrhages necessary for the titrations. One rabbit (B) gradually lost weight and died seventeen days after the second adrenal was removed, another (D) died of pneumonia thirty-six days after removal of the second adrenal. The rest of the animals are still living and apparently in good health (forty-two days after the total adrenalectomy). In the two animals that died autopsy failed to reveal any accessory adrenals on macroscopic examination. The animals which survived were later used for other experiments and killed.

In only one rabbit of the entire series (Rabbit F) there was found an accessory adrenal when the animal was used for another experiment on August 4th. The accessory gland was not seen at the time of the operation. It was located near the liver under the cava where it would have been seen if it were not very small. It is therefore quite certain that considerable hypertrophy of a very small accessory gland had taken place. The gland consisted only of cortical substance and weighed 0.150 gm. Prior to the first operation the animals were bled daily (4 to 5 cc.) and at intervals of two or three days to obtain their complement titer. The titrations were continued during the intervals between the two operations and following the second operation. Twenty-four hours following the operations the animals were bled. In no instance were the animals bled during the first few hours after first or second operation, to avoid possible factors of error. The titrations were done with the following quantities of serum: 0.025, 0.05, 0.075, 0.1, 0.125, 0.15, 0.175, and 0.2 cc. For accurate measurements the serums were diluted 1:2. The cell suspension used was 5 per cent sheep blood and the antsheep rabbit serum in doses

of 3 units. The sheep corpuscles were obtained from a laboratory sheep. All the rabbit serums were titrated immediately following the bleeding and separation of the serums. The period of incubation was one hour at 37°C. The tube that showed complete hemolysis was taken and recorded as the titer of that particular serum. The results are as follows:

Rabbit A, Control. Female. Weight, 2.77 kgm. On May 26, 1921, via abdominal route left nephrocapsulectomy was performed and visceral manipulation. The complement titer of this animal was very constant until June 11 when a drop was observed. The normal titer was 0.075 and this fell to 0.125 cc. but was again normal on June 17. On July 8, the titer was slightly stronger, namely 0.05 cc. These variations are normal and in part may also be due to changes of the cell suspension, etc. (See curve A.)

Rabbit B. Female. Weight, 2.81 kgm. On May 26, 1921, via abdominal route the right adrenal was excised. On June 8, 1921, the weight was 2.465 kgm. Via lumbar route (through the peritoneum) the left adrenal was excised. This adrenal appeared to be considerably hypertrophied. This animal died on June 25, 1921. Its weight was 1.95 kgm. Autopsy, negative. No accessory adrenals were found after a careful macroscopic examination. This animal having gradually lost in weight (860 grams) and the absence of any evidences of other obvious causes of death would indicate that death resulted from the absence of the adrenals. The curve of this animal was constant until June 3, that is, one week following the excision of the first adrenal, when a little drop was observed. The titer was again 0.05 cc. just prior to the second operation on June 8, and remained constant there after until June 17.

Rabbit C. Male, Weight, 2.59 kgm. On May 27, 1921, via abdominal route the right adrenal was excised. On June 8, 1921, his weight was 2.445 kgm. Via lumbar extraperitoneal route the left adrenal was excised. No hypertrophy of this gland was observed. Prior to the first operation there was a little variation in the titer of this animal which never varied beyond the range of 0.05 and 0.1 cc. Following the first operation a slight drop was observed from 0.05 to 0.075 cc. and following the second operation a less persistent but similar drop followed and on June 13 the titer was down to 0.05 cc. and on July 8 it was also 0.05 cc.

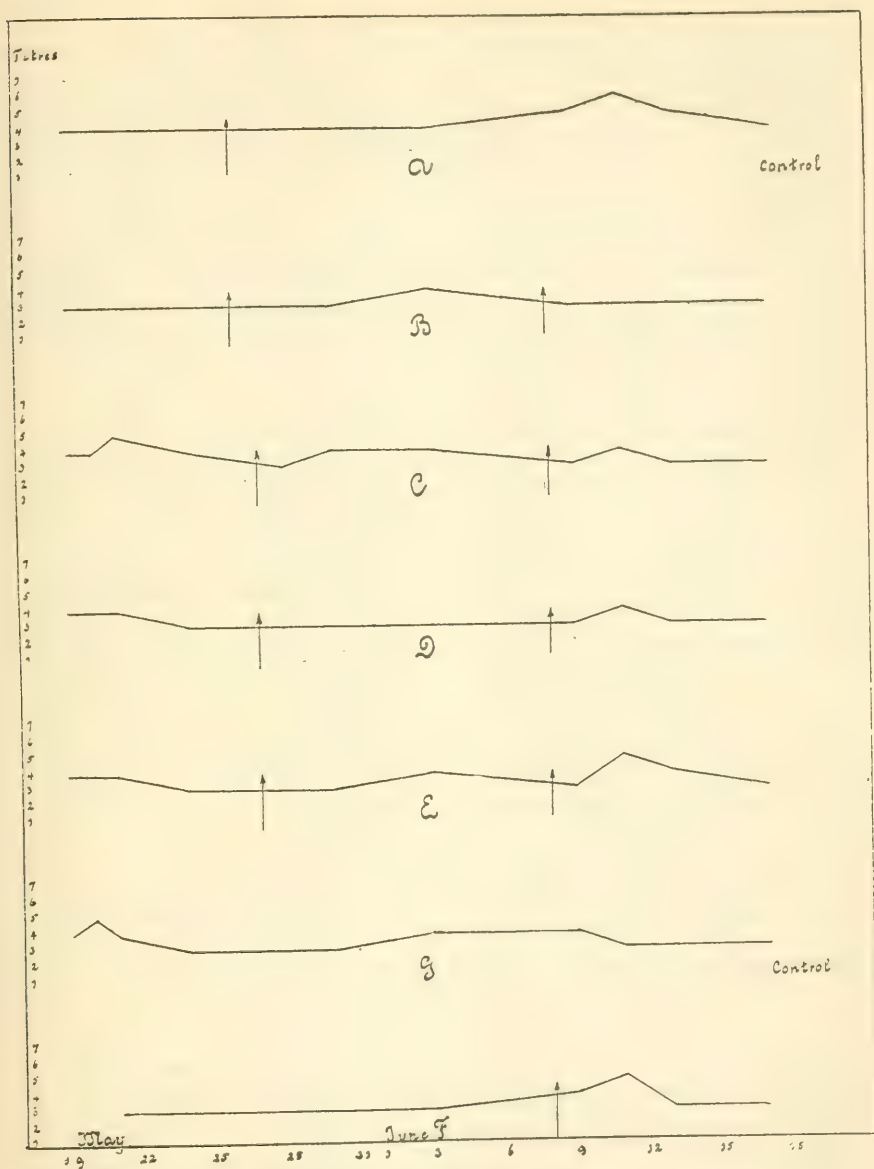


CHART SHOWING CURVES OF COMPLEMENT TITRATIONS

1 = 0.001 cc.; 2 = 0.025 cc.; 3 = 0.050 cc.; 4 = 0.075 cc.; 5 = 0.100 cc.; 6 = 0.125 cc.; 7 = 0.150 cc.; ↑ = dates of operations.

Rabbit D. Female. Weight, 2.73 Kgm. On May 27, 1921, the right adrenal was excised by abdominal route. On June 8, her weight was 2.52 Kgm. The left adrenal was then excised via the lumbar extraperitoneal route. The adrenal appeared to be only slightly hypertrophied. This animal died on July 14 with distinct consolidation of the lower lobe of the left lung. Her weight was 2.42 kgm. No accessory adrenals were found on macroscopic examination. Little variation in titer occurred in this animal. Prior to the first operation it was 0.05 cc., remained so during the interval between the two operations and fell slightly to 0.075 following the second operation, but was again normal on June 14. On July 8, it was also 0.05 cc.

Rabbit E. Male. Weight, 2.845 kgm. On May 27, 1921, via lumbar extraperitoneal route, the right adrenal was excised. On June 8, his weight was 2.5 kgm. and on this date the left adrenal was excised via the intraperitoneal route. This adrenal was not hypertrophied. This animal bled considerably from an anomalous vein on excision of one adrenal. The normal variations of the complement of this animal were between 0.05 and 0.075 cc. Following the second operation a drop to 0.1 cc. was observed which rapidly returned to normal on June 16. On July 8, the titer of this animal was 0.075 cc.

Rabbit F. Female, pregnant. Weight, 2.945 kgm. On June 8, 1921, via abdominal route a double adrenalectomy was performed. On June 12, the animal had a litter of 5 (not quite full term). Her titer was very constant, namely 0.05 cc. until June 3. Following the bilateral operation the titer dropped to 0.1 cc. and returned to normal on June 13. On July 8 it was 0.075 cc.

Rabbit G. Male. Weight, 2.755 kgm. This animal was not operated upon and was bled at the same time and equal amounts of blood drawn as in the case of the experimental animals. The titrations showed distinct variations that never went beyond the 0.1 cc. doses or below the 0.05 cc. doses (see curve G.)

The complement titers of these animals have been charted and clearly show that comparatively small variations occur not only following the operation but also in the control animals, indicating that the absence of the adrenal glands has no demonstrable influence on the complement activity. (See chart I).

SUMMARY

The complementing power of the blood serum of rabbits is not modified by unilateral or bilateral adrenalectomy. The variations occurring are normal because the same variations are observed in the control animals.

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HEREDITARY BLOOD QUALITIES

MEDICO-LEGAL APPLICATION OF HUMAN BLOOD GROUPING

REUBEN OTTENBERG

Adjunct Physician, Mount Sinai Hospital, Instructor in Bacteriology, College of Physicians and Surgeons, New York City

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Although the inheritance, according to certain perfectly definite mendelian principles, of group-specific substances in human blood has been known for ten years, the application of this information to medico-legal questions has not yet been made. It is the object of the present paper to present the possibilities of this practical application and to define the instances in which it can be used.

In 1908 while working on the relation of the isoagglutination to blood transfusion, I noticed that the groupings were hereditary and followed Mendel's law and made a brief statement to that effect in an article on the technic of the transfusion tests, read with Epstein before the New York Pathological Society (1). At that time I had examined only five families and was uncertain as to a part of the mechanism of the inheritance. For this reason and because of extraneous interruptions to my work, I postponed further publication.

In 1910 Von Dungern and Hirschfeld (2), in a splendid piece of work based on examination of 348 individuals belonging to 72 different families, completely proved the inheritance of these group-specific substances according to Mendel's law. The data which I had, agreed perfectly with, and were explained by their conclusions. And I mention my work not to claim priority over Von Dungern and Hirschfeld, but to justify my writing the present article which will be largely based on their observations.

In order to make what follows clear, it will be necessary first to go into an explanation of the main facts about iso-agglutination in human blood. The description of the technic of the test will be omitted since it can be found in any text book.

The term iso-agglutination is used to describe the agglutination of the red blood cells by contact with blood serum derived from another individual of the same species. This phenomenon, the prompt balling together into shapeless, tough, little masses of the previously smooth emulsion of red cells of one human being, when mixed with serum of another human being was described by Greenbaum (3) and by Shattuck (4) in 1900, and mistakenly supposed to be the result of disease. Landsteiner (5) in 1901 reduced its occurrence to a definite law. He found that with regard to the behavior of their serum and red blood cells, all human beings, without regard to race, sex or state of health, fall into one of three groups. In the first group the red cells are not agglutinable by any other human serum, while the serum is found to agglutinate the red cells of all persons not belonging to the first group. In the second group, the red cells are agglutinated by the serum of the first and third groups, while the serum agglutinates the cells only of the third group. The third is the obverse of the second group, its red cells being agglutinated by serum of the first and second groups, its serum agglutinating only cells of the second group. Landsteiner correctly concluded that the phenomena were due to the presence of two kinds of specific agglutinins of which one was present in the serum of the second group; another in the serum of the third group; and both in the serum of the first group. Landsteiner tested blood of mothers and their newborn children and found that these were frequently different from each other.

Descatello and Sturli (6) the following year confirmed Landsteiner's findings. Among 155 persons examined by them were 4 who did not fit in any of Landsteiner's three groups while their serum contained no agglutinin whatever. This fourth group was however, only definitely recognized and named as such in 1907 by Jansky (7). Descatello and Sturli, and later many other workers, showed that the group peculiarities are

permanent throughout life for each individual. They also made the important observation that in embryological development, the specific agglutinability of the red cells (called often agglutino-gen) appears first and is usually present at birth, while the specific agglutinative power of the blood serum (called agglutinin) which is to characterize the individual through life, may be absent at birth and may not appear for months or even several years.

W. M. Happ examined 131 infants and children and found that at birth and during the first month of life, agglutinin was rarely present, but by the first year the group was usually established and after two years, was always established as in adults (8).

If we represent the two red cell agglutinogens by A and B and the corresponding serum agglutinins by α and β , we can then schematically show the grouping of human blood according to Jansky as follows:

CHART 1

GROUP	SERUM	RED BLOOD CELLS
I	Agglutinates cells of the three other groups. Contains agglutinins α and β	Inagglutinable. Contain no agglutino-gen
II	Agglutinates cells of Groups III and IV. Contains agglutinin β	Agglutinated by serum of Groups I and III. Contain agglutinogen A
III	Agglutinates cells of Groups II and IV. Contains agglutinin α	Agglutinated by serum of Groups I and II. Contain agglutino-gen B
IV	No agglutinative effect. Contains no agglutinin	Agglutinated by serum of Groups I, II and III. Contain agglutinogens A and B

The grouping according to Jansky is logical and has priority. It has been adopted officially by the American Association of Immunologists and by the American Association of Pathologists and Bacteriologists. The groupings of Von Dungern and Hirschfeld and of Moss differ only in terminology. The reader must keep this in mind in going over the literature.

In 1902 Descatello and Sturli, and in 1907 Hektoen (9) definitely proved by the absorption method the existence of the two cell agglutinogens and two corresponding serum agglutinins postulated by Landsteiner. They showed that cells of group II, would absorb all the agglutinin from group III serum, but from serum of group I would absorb only the agglutinin which acts on group II cells leaving intact the agglutinin which acts on cells of group III. Likewise cells of group III will absorb all the agglutinin from group II serum, but from group I serum only the agglutinin for group III cells leaving the agglutinin which acts on group II cells unimpaired.

CHART 2

		NUM- BER OF CASES	I	II	III	IV
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Descatello and Sturli.....	Munich	155	42.6	37.4	17.4	2.6
Hektoen.....	Chicago	76	47	34	10	9
Moss.....	Baltimore	100	43	40	7	10
Von Dungern and Hirschfeld....	Heidelberg	348	36	47	11	5.7
Ottenberg*.....	New York	286	44	42	12	2

*Unpublished data.

In 1910 came the important work of Von Dungern and Hirschfeld already referred to. They showed that the susceptible substances (agglutinogens) A and B of the red cells never occur in a child if not present in one of the parents; that when one of these substances is present in both parents, it occurs in most of the children; while when a particular substance (A or B) is present in only one parent, some of the children inherit it; when neither parent has a particular one of these substances, no child ever shows it. In other words A as an inherited character is dominant over the character absent A (not A), while B is dominant over absent B (not B), and the two pairs of unit characters, A and Not A, B and Not B are inherited independent of each other. Medico-legally then, if A or B is present in a child's blood one of the alleged parents must possess it.

While the rough proportions of individuals belonging to the four different groups have been about the same in the hands of

all workers, the actual percentages have shown considerable variations in different places.

The key to these differences as well as much new light on the possible origin of these blood peculiarities is found in a fascinating recent piece of research by Hirschfeld. Working in Serbia during the war, Ludwick and Hanka Hirschfeld (10) did many thousand iso-agglutination tests on men of different races and classified all according to whether their red blood cells contained A or B or neither. They found in general that all human races present some A and some B, but that there is a great preponderance of A in European, of B in Asiatic and African races. Thus Englishmen showed 464 A to 102 B (a ratio of 82 per cent A: 18 per cent B), while natives of India showed 273 A to 437 B, (a ratio of 38 per cent A: 62 per cent B). Certain races geographically intermediate, Arabs, Turks, Jews and Russians showed also an intermediate numerical ratio, i.e., about equal proportions of A and B. These facts strongly suggest that the original human race was homogeneous, i.e., showed neither A nor B, that somewhere probably in the central plateau of India during prehistoric times there arose a sport or mutation, B in the biochemical structure of the red cells, while somewhere toward the north of Europe there arose another mutation, A. The present geographical distribution of A and B is then accounted for by their mode of inheritance and the wandering and intermingling of the races.

Let us now turn to consider the mechanism of heredity of these blood properties. It would be equally possible, and would lead to the same final result to regard as unit characters either the serum agglutinins or the susceptible substance in the red cells, since with perfect regularity, agglutinin α occurs in the serum of any individual whose red cells do not possess A, and β occurs in the serum of one whose red cells have no B. But since the susceptible substances in the individual develop first, and since the work of Von Dungern and Hirschfeld is in terms of A and B, we will adhere to these terms.

The quality A is dominant to Absent A, and B to absent B. The qualities Absent A and Absent B (which for brevity I will

call Not A and Not B) are not mere blanks but are definite qualities of red cells associated respectively with the development in the serum of agglutinins α and β . Von Dungern and Hirschfeld showed that the inheritance of the paired qualities A and Not A, B and Not B have no relation to each other. Thus, if we compared A and Not A with Black and White, B and Not B could be represented by some entirely different and independent pair of characters, like Tall and Short.

Now with regard to the inheritance of A or Not A, there are for each individual three possibilities: (1) either both of his parents are A and the individual is pure A and can only transmit to his offspring the quality A, since all of his germ cells must carry that quality; (2) or both of his parents are Not A and the individual is pure Not A and can only transmit Not A; or (3), one parent is A, the other Not A, and the individual is a hybrid, A-Not A, and, (according to the mendelian law) his germ cells in equal numbers carry the properties A and Not A, even though the red cells of the individual himself show (due to the dominance of that quality), only A.

With regard to the inheritance of B or Not B, exactly the same three possibilities exist.

If we represent apparent or dominant qualities by capital letters, recessive ones by small letters, and abbreviate Not A, Not B to NA, NB, na, nb, we can then represent the hereditary constitution of the four classes of human blood as shown in chart 3.

It is clear at once that group I (the most numerous group representing about 45 per cent of the community) can never be hybrid. Group II (the next most numerous, representing about 40 per cent of the community) can only be hybrid with regard to its dominant quality A, so that there are two kinds of persons belonging to group II, pure A-A, transmitting only A to offspring, and hybrid, A-Not a, transmitting these two qualities to offspring in equal numbers. The same is true for group III, (12 to 15 per cent of the community); it can only be hybrid with regard to its dominant quality B. Group IV on the other hand, (the rarest of the groups, representing only 2 to 5 per cent of the community) has four possibilities; it may be pure with

Group I

NA	NA
NB	NB

Group II

A	A
NB	NB

Pure

A	na
NB	NB

Hybrid

Group III

NA	NA
B	B

Pure

NA	NA
B	nb

Hybrid

Group IV

A	A
B	B

Pure

A	na
B	B

Partial Hybrid

A	A
B	nb

Partial Hybrid

A	na
B	nb

Full Hybrid

CHART 3. HEREDITARY CONSTITUTION OF RED CELLS OF THE FOUR GROUPS OF HUMAN BLOOD

regard to both dominant qualities A and B, or pure with regard to one, while hybrid with regard to the other, or hybrid with regard to both. For further analysis, I have numbered these four different types of group IV, 1, 2, 3, and 4.

Let us now consider in turn the offspring of unions of a member of one group with another member of the same group, and then the offspring of unions of two different groups. The first possibility is the union of a person belonging to group I with another belonging to group I. These, while the most numerous, are the simplest of all (chart 2). The offspring are all and uniformly group I.

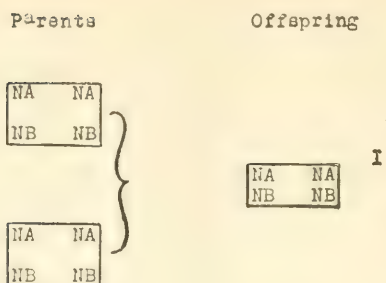


CHART 4. HEREDITARY POSSIBILITIES IN UNIONS OF GROUP I WITH GROUP I

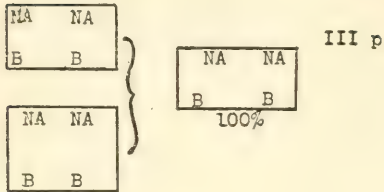
Next come unions of two persons both belonging to group II. These are third in frequency (the second in frequency being unions of I and II). There are three possibilities, union of (a) pure II with pure II; (b) pure II with hybrid II; and (c) hybrid II with hybrid II. (chart 3). Of these (a) can result only in pure II, (b) in equal numbers of pure and hybrid II, and (c) in pure II, hybrid II, and in I in the ratio 1:2:1. From unions of II with II, then, there are only two possible kinds of children, II and I. And on the average (if the numbers of pure II and hybrid II in the community are equal) the proportion of II to I among the offspring of such unions will be as eleven to one, (or eighty-three per cent to seventeen per cent), as can be easily seen from chart 3. Unions of II and II can never produce III or IV.

The unions of group III with group III follow the same rule as those of group II with group II and produce only III and I, and in the same ratio eleven to one. (chart 6). They can never produce offspring belonging to group II or IV.

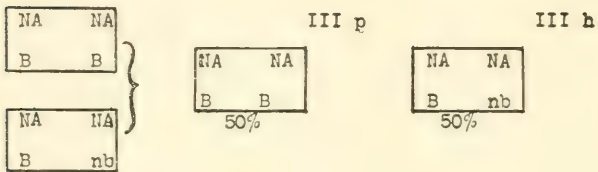
The unions of group IV with group IV, though the rarest of all possible unions, present the greatest number of possible combinations, due to the fact that beside pure group IV there are three different varieties of hybrids. Of the resulting offspring the great majority, as can be seen from chart 7, are group IV, a few are II or III, and an exceedingly small proportion (about 1 per cent), group I.

ParentsOffspring.

- a) First possibility
Pure



- b) Second Possibility
Pure-Hybrid



- c) Third Possibility
Hybrid-Hybrid

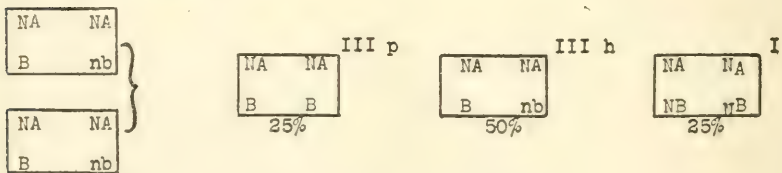


CHART 6. UNIONS OF III WITH III

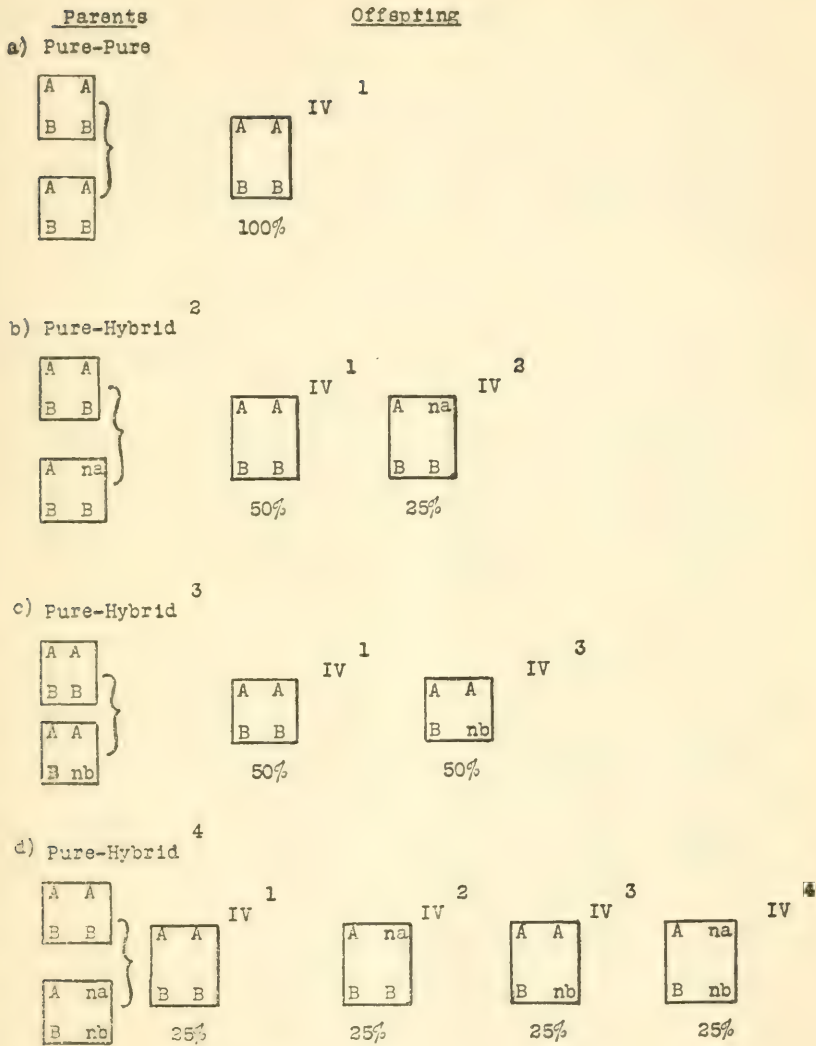
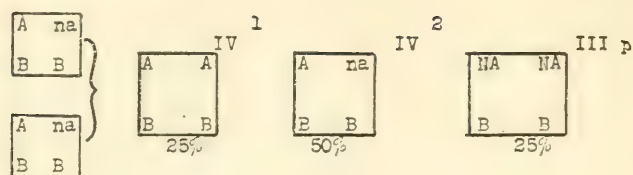
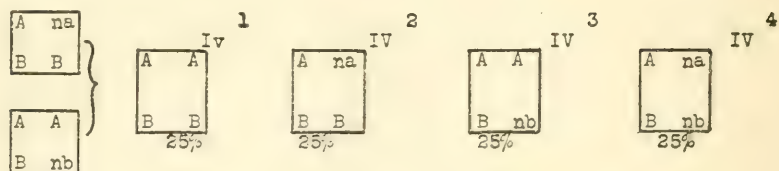


CHART 7. UNIONS OF IV WITH IV

e) Hybrids 2 and 2



f) Hybrids 2 and 3



g) Hybrids 2 and 4

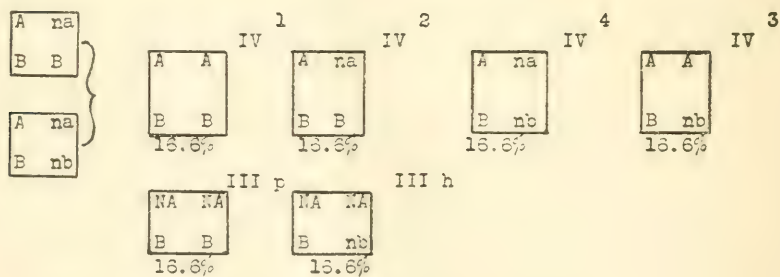
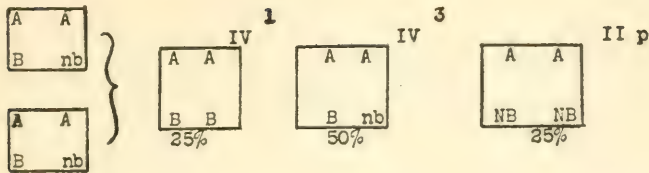
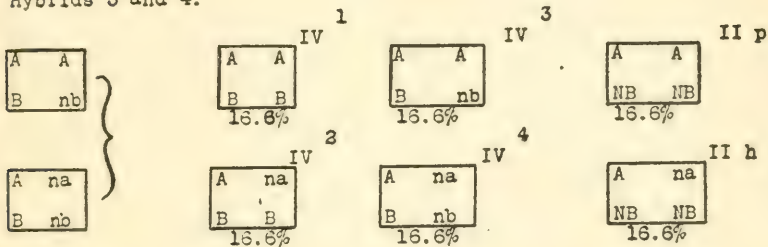


CHART 7 (continued). UNIONS OF IV WITH IV

h) Hybrids 3 and 3.



i) Hybrids 3 and 4.



j) Hybrids 4 and 4

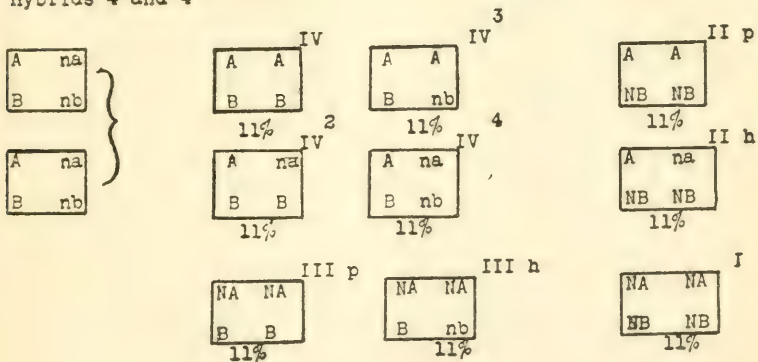


CHART 7 (continued). UNIONS OF IV WITH IV

Parents
a) Pure I and II

NA	NA
NB	NB

A	A
NB	NB

Offspring

II h

A	na
NB	NB

100%

b) I and Hybrid II

NA	NA
NB	NB

A	na
NB	NB

I

NA	NA
NB	NB

50%

II h

A	na
NB	NB

50%

CHART 8. UNIONS OF I AND II

Parents
a) Pure I and III

Offspring

NA	NA
NB	NE

NA	NA
B	B

III h

NA	NA
B	nb

100%

b) I and hybrid III

NA	NA
NB	NE

NA	NA
B	nb

I

NA	NA
NB	NB

50%

III h

NA	NA
B	nb

50%

CHART 9. UNIONS OF I AND III

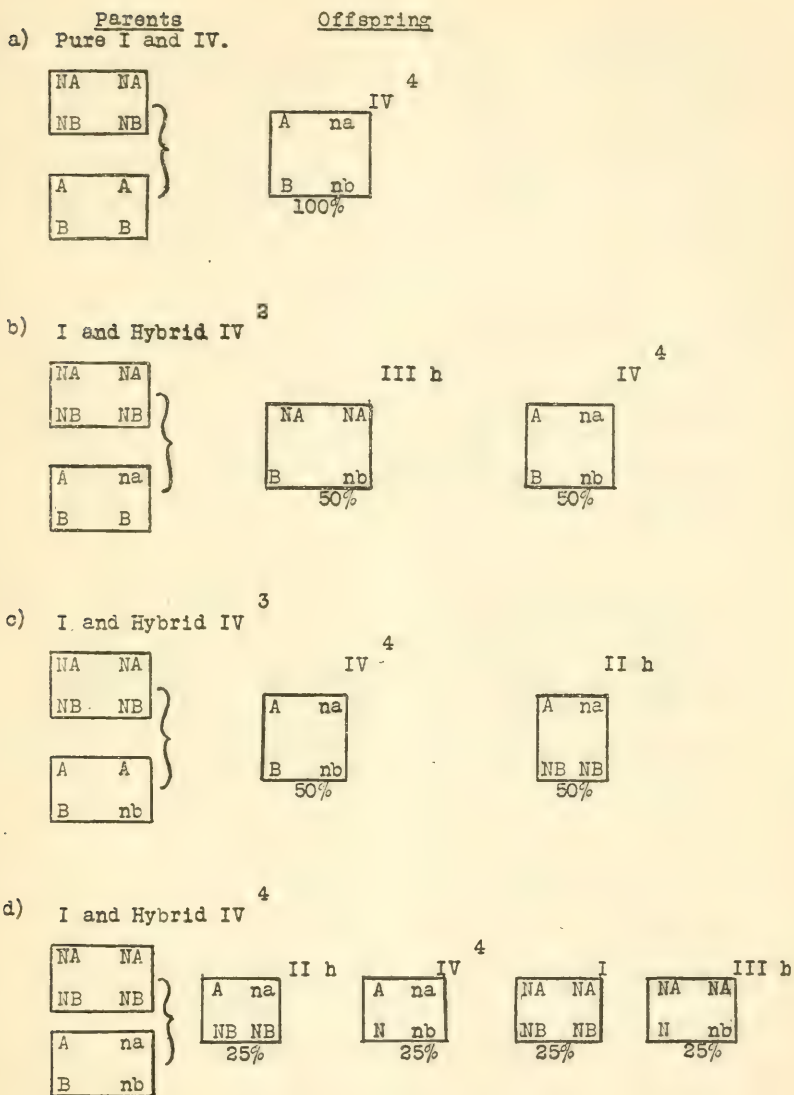


CHART 10. UNIONS OF I AND IV

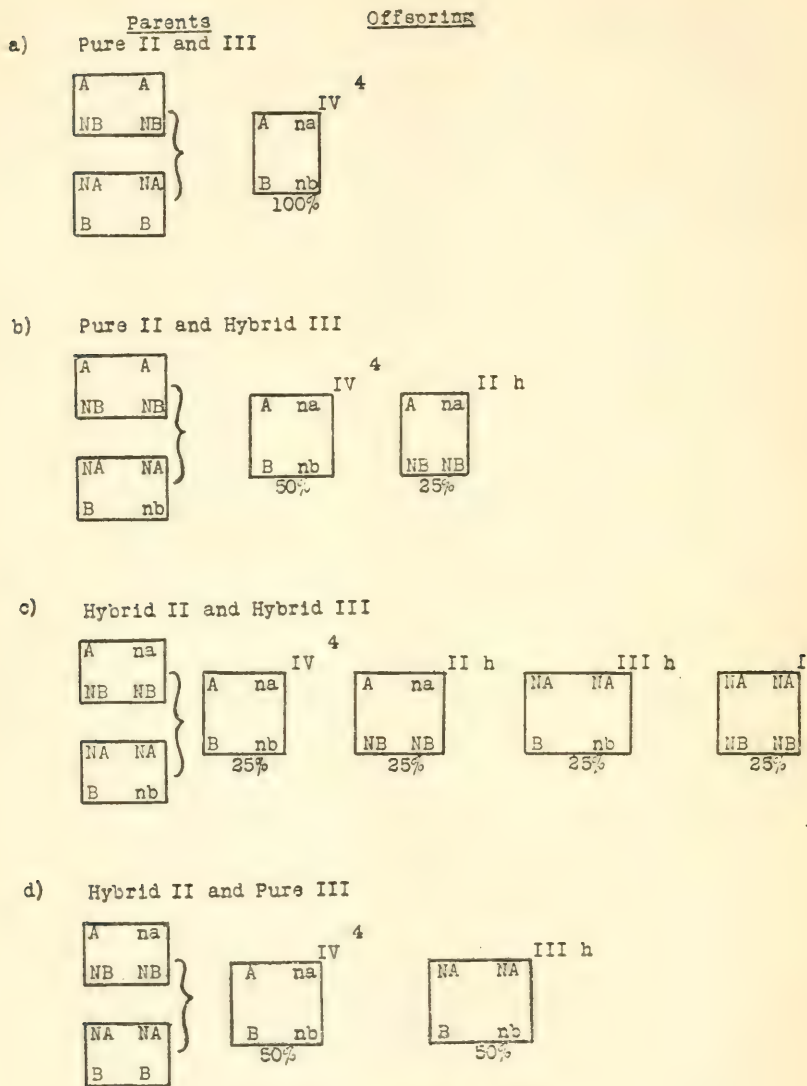


CHART 11. UNION OF II AND III

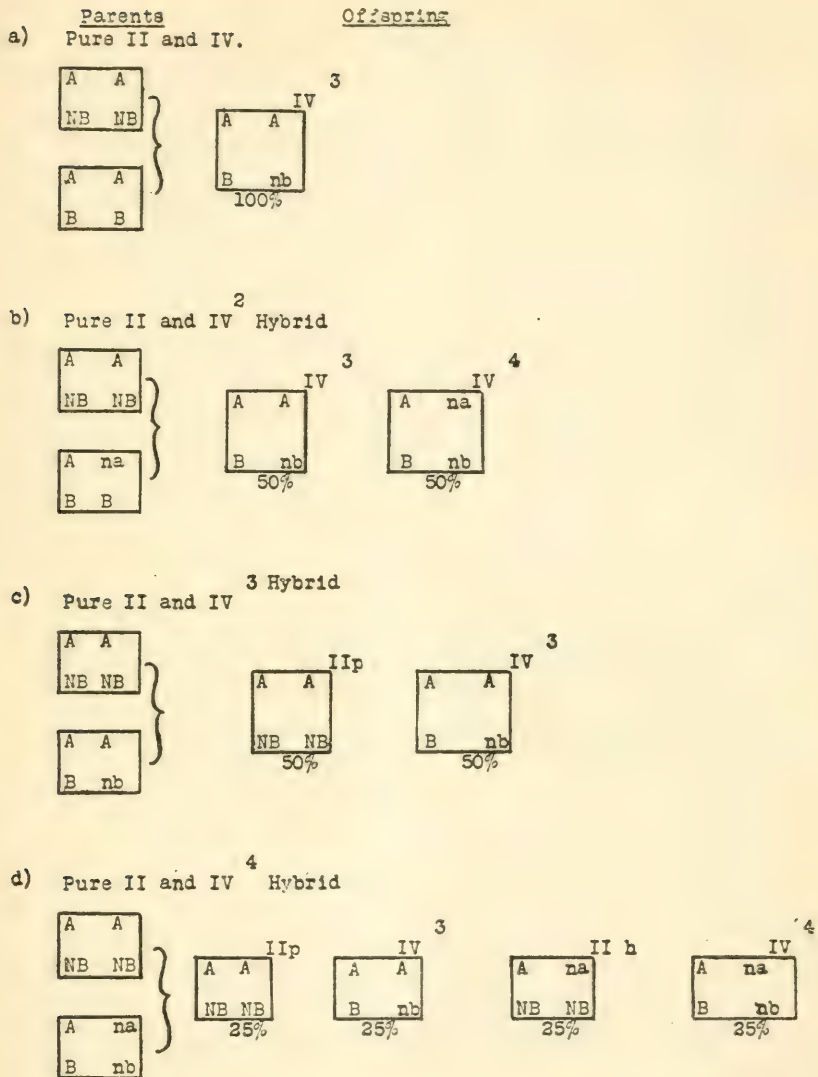
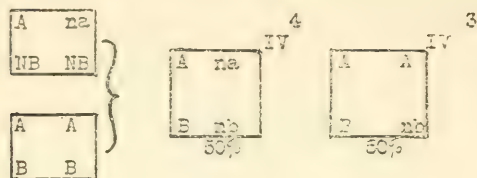
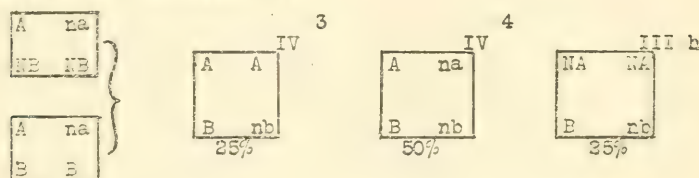


CHART 12. UNIONS OF II AND IV

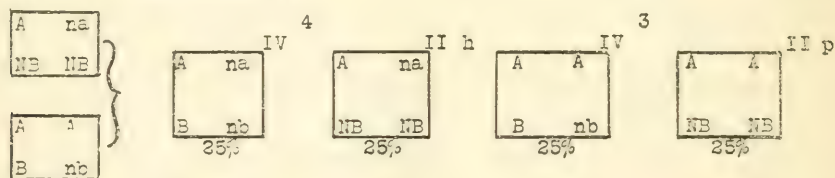
e) Hybrid II and Pure IV.



f) Hybrid II and Hybrid IV



g) Hybrid II and Hybrid IV



h) Hybrid II and Hybrid IV

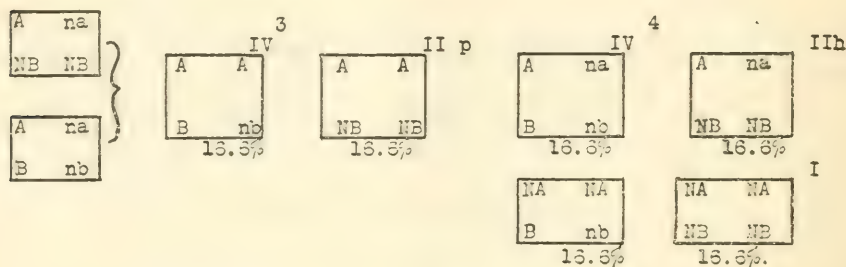


CHART 12. (continued). UNIONS OF II AND IV

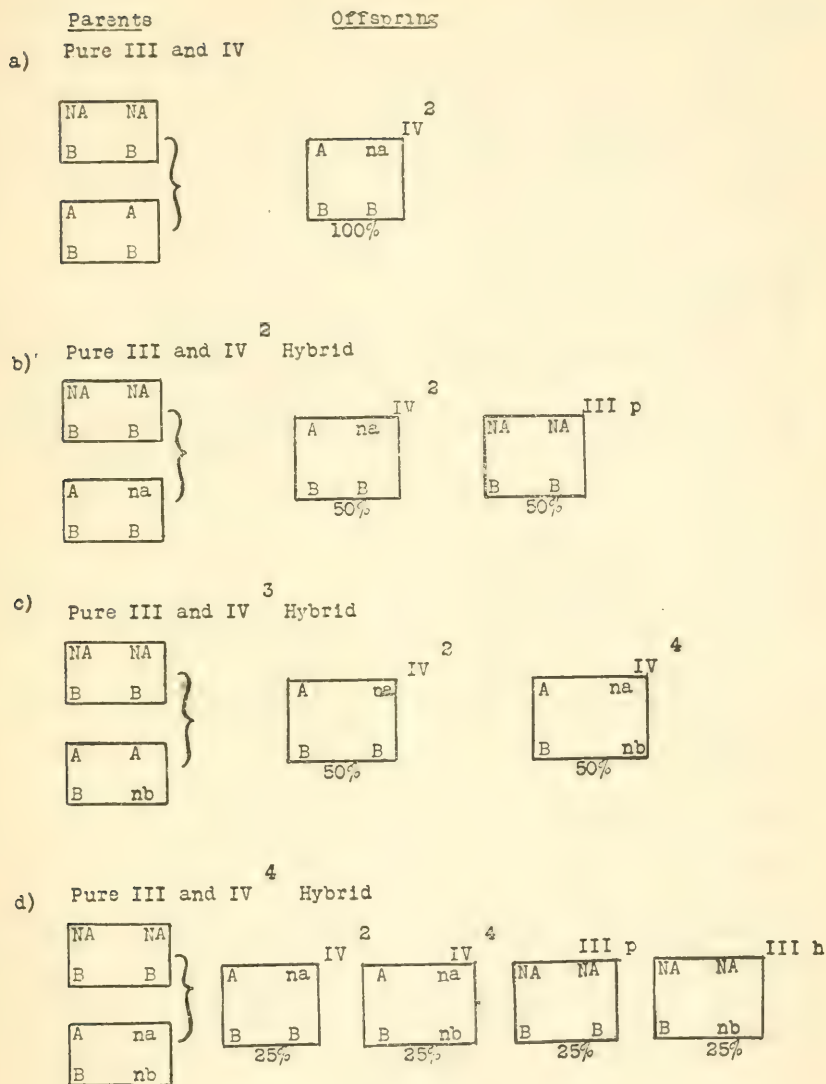
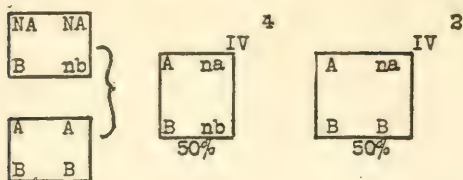
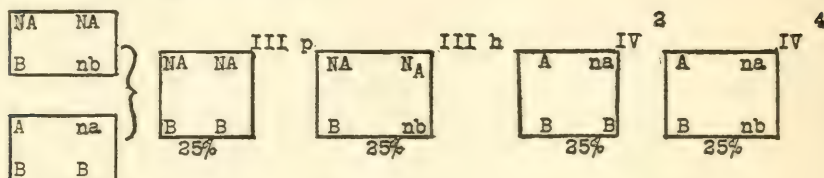


CHART 13. UNIONS OF III AND IV

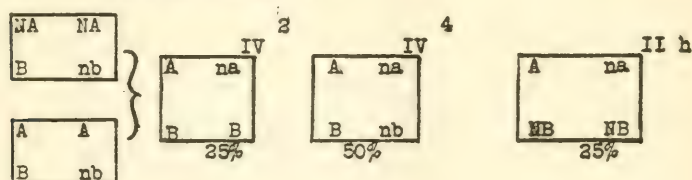
e) Hybrid III and Pure IV.



f) Hybrid III and Hybrid IV



g) Hybrid III and Hybrid IV



h) Hybrid III and Hybrid IV

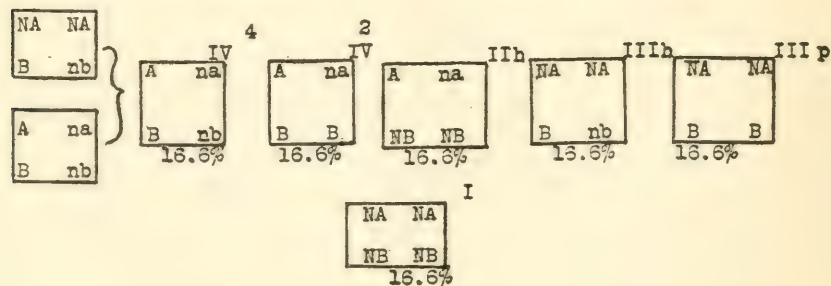


CHART 13. (continued). UNIONS OF III AND IV

Turning now to unions between members of two different groups, the most frequent of these (and the second most frequent in occurrence of all possible unions) is that between I and II. The result here (chart 8) is simple; the offspring are all the same as the parents, I or II, (and in the ratio 25 per cent I to 75 per cent hybrid II, provided that pure and hybrid II are equally numerous among the parents).

The result of the relatively rarer unions of groups I and III are similar. All the offspring are I or III and again in the ratio of one to three (chart 9).

Unions of I and IV (chart 10) like those of II and III (chart II) can result in offspring of all four classes.

Unions of groups II and IV (chart 12) result chiefly in groups II and IV, but also in a small proportion (about 2 per cent) of III and a still smaller proportion of I.

Likewise the very rare unions of III and IV (chart 13) result chiefly in III and IV but also in about 2 per cent of II and 1.5 per cent of I.

It is possible now to define the limited circumstances under which our knowledge of the heredity of substances A and B can be useful.

We summarize the results above in chart 14.

CHART 14

Unions of I and	I	give only I
Unions of I and	II	} give only I and II
Unions of II and	II	
Unions of I and	III	} give only I and III
Unions of III and	III	

The unions tabulated in this chart, which comprise over 80 per cent of all unions, are those in which the kind of possible offspring is definitely limited, and these therefore are the instances which may, under certain circumstances, be of medico-legal value.

On the other hand, all unions containing a member of group IV and unions of II and III, may give rise to offspring of any of the four groups. Likewise a child of group I may result from any combination of parents.

Suppose then that the blood of a child and the alleged parents have been tested, what conclusions can be drawn? If the child's blood is the correct group for the alleged parents, then we can say that the child *could* be their offspring, not that it must of necessity be. But on the other hand, if the child's group is wrong for the two asserted parents, then one can say with absolute certainty that the child must have a parent other than one of those asserted.

The commonest instance of course is that of disputed paternity. Here we can readily tabulate the instances in which it is possible to be sure that the child is illegitimate or is not the child of an asserted father (chart 15).

CHART 15

Instances in which the child must be illegitimate, or not the child of the supposed father

KNOWN MOTHER	SUPPOSED FATHER	CHILD
I	I	II, III, IV
I	II	III, IV
I	III	II, IV
II	I	III, IV
II	II	III, IV
III	I	II, IV
III	III	II, IV

The same kind of evidence (chart 15) can be used, either to prove the illegitimacy of the offspring or (circumstances being reversed) to prove the innocence of a correspondent asserted to be the father of a given child.

Likewise, in the rarer cases of disputed maternity or of alleged substitution of one child for another, chart 15 shows the instances in which it can be stated with certainty that the child is spurious; i.e., a child of one of the groups in the third column cannot be the offspring of the parents on the corresponding lines in the first two columns.

In infants and very young children the test can only be relied upon if it shows definite group characteristics, which it does in

the majority of cases. In all cases not only of children but of adults, the individual's blood serum should be tested for its effect on the red cells of known group II and III persons in order further to confirm the grouping determined by testing his red cells with known group II and III serum. This requires about 1 cubic centimeter of blood which can be taken from an arm vein.

In practice, of course, it may be difficult to obtain the consent of all three parties, (or at times four), to the blood test. The test can be easily done with a few drops of blood obtained from a painless prick with a small needle. Considering this, and the importance of the questions often at issue, it seems as though some legal means could be devised by which the persons concerned could be compelled to allow the examination at the hands of a representative of the court.

CONCLUSION

The number of instances in which the group blood test is of value is limited, but within these limits its evidence is conclusive.

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THE REACTION OF THE RAT TO DIPHTHERIA TOXIN

WITH OBSERVATIONS ON THE TECHNIC OF THE ROEMER METHOD OF TESTING DIPHTHERIA TOXIN AND ANTITOXIN

ARTHUR F. COCA, ERNEST F. RUSSELL AND WILLIAM H. BAUGHMAN

*From the Department of Bacteriology of Cornell University Medical College and
the Second Medical Division of the New York Hospital*

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It has been the practice of one of us to demonstrate to students the natural immunity of the rat to diphtheria toxin. This was done by injecting intraperitoneally 1 cc. of diphtheria toxin possessing a minimal lethal dose of 0.001 cc. Two rats were injected. One of these was kept under observation and always survived; the other was killed by bleeding on the morning following the injection and the whole quantity of the serum obtained was injected into a guinea-pig to demonstrate in the serum the presence of free toxin. The guinea-pig regularly died. This experiment showed that the immunity of the rat is not due to antitoxin in the animal's blood.

It was thought at first that since the rat can survive the injection of 1000 times the minimal lethal dose of toxin for the guinea-pig it must be absolutely immune to the toxin. Under this impression experiments were carried out to see whether the rat is capable of antitoxin production, as antitoxin production has not been demonstrated in animals that are absolutely immune to the respective toxin.

In examining the serum of the rats for the presence of antitoxin we have employed the method of Roemer which is adapted to the detection of very small quantities of toxin or antitoxin. In the course of our experience with this method some technical difficulties were encountered.



FIGURE 1

The first difficulty had been met in preliminary experiments in the performance of the intracutaneous injection. In these experiments we had shaved the entire abdomen with a sharp razor—avoiding trauma—and had introduced the needle (27 gauge) into the somewhat tightly drawn skin in the manner employed for the Schick injection. With this manner of injection we were quite unable to control the depth of the injection. Before we began the present series of experiments we had learned from Dr. Abraham Zingher a method of injection, which, when properly applied, insures a truly superficial site of the injection. This method, which Dr. Zingher has not published, is illustrated in figure 1. The shaved skin is taken up in a loose fold over the forefinger and the needle is inserted superficially near the free border of the fold. The injection of 0.1 cc. of fluid in this situation always results in the formation of a tense vesicular swelling with a sharply defined base.

The second difficulty lay in the fact that occasionally in our experience the injection of identical material in different animals or even in different sites in the same animal produced widely different effect; for example, necrosis in one place, no necrosis in another.

In table 1 are shown the results of the preliminary test of the toxicity of the toxin with the use of the Roemer method.

It is seen that although in most of the animals necrosis was produced by both of the toxin dilutions, in guinea pig-285 this effect was lacking in one site even with the 1:600 dilution.

With this possible source of error in mind we conducted the earlier experiments with the use of duplicate injections of all mixtures into two animals. In accordance with the standard adopted for the usual subcutaneous method of injection, guinea-pigs had been selected weighing from 250 grams to about 300 grams, which is about the range of weight mostly observed by Roemer (1) in his original experiments. On account of the difficulty of maintaining a supply of animals of the selected range of weight some tests were carried out to see whether heavier animals could be used. In the first series of these tests the injections shown in table 1 were duplicated in six guinea-pigs

weighing between 430 and 630 grams. In this series there were no irregular results. Every injection produced necrosis. As it seemed possible that this somewhat surprising result was due to a greater susceptibility to the toxin on the part of the skin of the heavier animals, a further quantitative comparison was made, the results of which are presented in table 2.

It is seen that whereas in the heavier animals necrosis was regularly produced by 1/24,000 cc. of the toxin, in the smaller

TABLE 1

*Preliminary test of the toxicity of diphtheria toxin with the use of Roemer's method.
Result on the fourth day after intracutaneous injection of 0.1 cc.*

GUINEA-PIG 289; WEIGHT 290 GRAMS				GUINEA-PIG 285; WEIGHT 260 GRAMS			
L.P.	1 : 900	Necrosis	6 mm.	L.P.	1 : 900	Necrosis	7 mm.
R.P.	1 : 900	Necrosis	3 mm.	R.P.	1 : 900	0	
L.A.	1 : 600	Necrosis	10 mm.	L.A.	1 : 600	0	
R.A.	1 : 600	Necrosis	10 mm.	R.M.	1 : 600	Necrosis	10 mm.
GUINEA-PIG 286; WEIGHT 260 GRAMS				GUINEA-PIG 1849; WEIGHT 250 GRAMS			
L.P.	1 : 90	Necrosis	3 mm.	L.P.	1 : 900	Necrosis	2 mm.
R.P.	1 : 90	Necrosis	3 mm.	R.P.	1 : 900	Necrosis	3 mm.
L.A.	1 : 600	Necrosis	7 mm.	L.A.	1 : 600	Necrosis	7 mm.
R.A.	1 : 600	Necrosis	7 mm.	R.A.	1 : 600	Necrosis	8 mm.
GUINEA-PIG 290; WEIGHT 250 GRAMS				GUINEA-PIG 479; WEIGHT 310 GRAMS			
L.P.	1 : 900	Necrosis	7 mm.	L.P.	1 : 900	Necrosis	10 x 3 mm.
R.P.	1 : 900	Necrosis	7 mm.	R.P.	1 : 900	Induration	
L.A.	1 : 600	Necrosis	10 mm.	L.A.	1 : 600	Necrosis	7 mm.
R.A.	1 : 600	Necrosis	10 mm.	R.A.	1 : 600	Necrosis	10 mm.

animals, corresponding with the earlier experiences, irregularity of effect was obtained with as much as 1/9000 cc.

It is evident in these experiments that the skin of the older guinea-pigs is more sensitive to the action of diphtheria toxin than the skin of the younger animals. For this reason the heavier animals must be preferred as test animals for the intradermal method. In numerous subsequent experiences this conclusion has been confirmed.

In examining the serum of the rats for the presence of anti-toxin the following procedure has been employed:

Serum was obtained by centrifugating the defibrinated blood and it was heated for thirty minutes at about 54°C. The toxin used throughout this study was furnished by Dr. Edwin J. Banzhaf of the Board of Health of New York City. The minimal lethal dose of this toxin was 1/400 cc. and the L_+ dose was 0.18 cc.

The toxin was used in a constant quantity in all of the mixtures; namely, 0.2 cc. of a 1 : 120 dilution. This quantity was somewhat less than 1/100 of the L_+ dose. Hence, failure of any certain quantity of serum to neutralize the toxin demonstrated that that quantity of serum contained not more than 1/100 of an antitoxin unit.

TABLE 2

Influence of the age of the guinea-pig on the result of Roemer's test for diphtheria toxin
Result on fourth day after intracutaneous injection of 0.1 cc.

TOXIN DILUTION	GUINEA-PIG 213, WEIGHT 630 GRAMS	GUINEA-PIG 259, WEIGHT 290 GRAMS	GUINEA-PIG 280, WEIGHT 460 GRAMS	GUINEA-PIG 265, WEIGHT 240 GRAMS
1 : 2400	Necrosis	Scaling	Necrosis	Induration
1 : 1800	Necrosis	Scaling	Necrosis	Slight scaling
1 : 1500	Necrosis	Induration	Necrosis	Necrosis
1 : 900	Necrosis	Necrosis ?	Necrosis	Necrosis
TOXIN DILUTION	GUINEA-PIG 289, WEIGHT 580 GRAMS	GUINEA-PIG 286, WEIGHT 220 GRAMS	GUINEA-PIG 281, WEIGHT 550 GRAMS	GUINEA-PIG 283, WEIGHT 260 GRAMS
1 : 2400	Necrosis	Slight scaling	Necrosis	Scaling
1 : 1800	Necrosis	0	Necrosis	Slight necrosis
1 : 1500	Necrosis	Some scaling	Necrosis	Slight necrosis
1 : 900	Necrosis	Necrosis	Necrosis	0

The volume of the mixtures was always 1 cc. The quantity of mixture injected was always 0.1 cc. The animals were generally under daily observation and the final record of the results was made on the fourth day.

A number of preliminary tests had shown that in 0.8 cc. of normal rat's serum there is not enough antitoxin to prevent the necrotizing action of 0.2 cc. of a 1:120 dilution of the toxin. Following is a protocol of the experiments designed to determine whether the injection of diphtheria toxin into the rat results in antitoxin production.

August 11. Eight white rats receive 0.5 cc. of diphtheria toxin each by subcutaneous injection.

August 18. The eight rats injected on August 11 receive exactly similar injections.

August 25. Six of the injected rats receive a third injection exactly similar to the first. The other two rats are etherized and bled to death from the heart. The defibrinated blood is pooled and the serum, obtained by centrifugation, is heated for 30 minutes at 54°C . 0.8 cc. of this serum did not interfere with the necrotizing action of 0.2 cc. of toxin diluted 1:120.

September 1. One of the injected rats is bled and the heated serum is examined as usual. No antitoxic action is demonstrable. The remaining rats receive the fourth injection of 0.5 cc. of toxin.

September 8. One of the injected rats is bled and the heated serum is examined. 0.4 cc. of this serum are found to prevent completely the necrotizing action of 0.2 cc. of toxin (1 : 120). Smaller quantities of the serum are not tested. The remaining rats receive the fifth injection of 0.5 cc. of toxin.

September 15. The serum of one of the injected rats is obtained and tested as usual. One-tenth of a cubic centimeter of the serum is found to neutralize completely 0.2 cc. of toxin (1 : 120). 1/40 cc. exhibited no neutralizing effect. The remaining rats receive the sixth injection of 0.5 cc. of toxin.

September 22. The serum of one of the injected rats was obtained and heated as usual. 1/40 cc. of this serum prevented the necrotizing action of the usual quantity of toxin but allowed some sealing. 1/160 cc. of the serum exhibited no antitoxic power.

These experiments show that the white rat, although insensitive to the injection of 1000 times the lethal dose of diphtheria toxin for the guinea pig can produce antitoxin after four injections of 200 doses. After the sixth injection the serum of the last rats examined contained at least 4/10 of an antitoxin unit per cubic centimeter.

The foregoing experiments seemed to indicate that an animal can produce antitoxin against a toxin to which it is not susceptible. It did not seem likely that an animal which was able to receive 1000 lethal doses of toxin without exhibiting symptoms would be intoxicated by a larger quantity of the toxin.

Nevertheless, the following tests were carried out as a precautionary measure:

October 18. 14 cc. of diphtheria toxin had been mixed with 0.2 cc. (100 units) of diphtheria antitoxin and the mixture had been kept in the ice-box. 4 cc. of this overneutralized toxin were injected subcutaneously into each of three normal white rats. Three other normal rats received 4 cc. of each of toxin with which no antitoxin had been mixed. All of the six animals were etherized, as usual, before the injections, but they did not recover as quickly as usual and all of them exhibited twitchings of the whole body for a considerable time. Eventually, however, all recovered and on the following day they seemed normal, with the exception of one of the animals that had received the unneutralized toxin. This animal died during the day. Autopsy showed slightly congested adrenals and some excessive fluid in the abdominal cavity.

The other two animals that had received the unneutralized toxin died on October 21 and 22 and both presented slightly but distinctly congested adrenal glands. All of the rats that received the neutralized toxin survived without symptoms.

The foregoing experiment demonstrates that the rat is not entirely immune to diphtheria toxin: it demonstrates a slight degree of susceptibility which corresponds with the slight power of antitoxin production in this animal.

THE MECHANISM OF THE NATURAL RESISTANCE OF THE RAT TO DIPHTHERIA TOXIN

Our knowledge regarding the natural cellular immunity to toxin is limited almost entirely to the assumption of Ehrlich that immune cells lack the appropriate "receptors" through which alone, according to him, toxin can become effectively attached to cells. However, in the one instance in which it has been possible to put this assumption to experimental test the theory has been shown to be inapplicable.

Suspended in a medium containing electrolytes, the red blood corpuscles of the ox and the sheep are entirely immune to the action of cobra hemotoxin. If these corpuscles are suspended in isotonic solutions of sugars (2) in the complete absence of elec-

trolytes they are found to be susceptible to cobra hemotoxin. The simple addition of electrolyte to the sugar medium restores the cellular immunity.

It has been found that in a medium containing electrolytes the resistant corpuscles are unable to absorb the cobra hemotoxin, whereas in a medium free from electrolytes absorption of a considerable quantity of hemotoxin can be demonstrated.

These facts show that the immunity of the resistant corpuscles is due to a physical condition of the corpuscular substance, which prevents the entrance of the toxin. This physical condition is altered by the mere exclusion of electrolytes, by which means the corpuscles become permeable to the toxin.

The assumption suggested itself that the mechanism of the natural corpuscular resistance to cobra hemotoxin is operative in all instances of natural cellular immunity to toxin, including, therefore, that of the rat to diphtheria toxin. The truth of this assumption could not be tested in the present instance by the exclusion of electrolytes in the animal experiment—it was impossible to find out whether in the absence of electrolytes the rat's tissues would become more susceptible to the action of diphtheria toxin.

However, evidence regarding the mechanism of the rat's immunity could be sought by another means. It was evident that if a fixed quantity of toxin be injected into an animal the concentration of the toxin in the body fluids will be affected according to whether the toxin permeates the cells or not. This principle has been used by Hedin (3) and later by Kosakai (4) to determine the permeability of blood corpuscles for various substances; it was applied in the present study as follows:

A certain quantity of diphtheria toxin per 100 grams of animal weight was injected intraperitoneally into a rat and into a guinea-pig and after six to seven hours the animals were etherized and the toxin content of their serum was determined by subcutaneous injection of the sera into guinea-pigs.

If the rat's cells are only slightly permeable to the toxin so that most of the toxin remains in the body fluids the serum of the

rat obtained in this experiment should be more toxic than that of the guinea-pig, whose cells are highly susceptible and therefore easily permeable to the toxin. The results of the experiments presented in the following protocols amply meet these hypothetical conditions.

Protocol 1

Rat 1, weighing 250 grams and guinea-pig 1, weighing 220 grams, received respectively 2.5 cc. and 2.2 cc. of diphtheria toxin by intraperitoneal injection. Six hours later both animals were etherized and bled from the heart. The sera, obtained by centrifugation of the defibrinated blood were injected subcutaneously into normal guinea-pigs as follows:

GUINEA-PIG NUMBER	WEIGHT	MATERIAL INJECTED	QUANTITY INJECTED	RESULT
	<i>grams</i>		<i>cc.</i>	
265	250	Serum rat 1 (1 : 20)	1.0	Died in 4 days
256	250	(Same)	0.5	Local edema: S.
273	210	Serum guinea-pig 1 (1 : 20)	2.0	Died in 3 days
274	240	(Same)	1.0	Local edema: S.

S = survived.

Protocol 2

Rat 2, weighing 310 grams and guinea-pig 2, weighing 275 grams, receive respectively 1 cc. and 0.9 cc. of diphtheria toxin by intraperitoneal injection. Six hours later the animals were bled and the serum obtained was injected into guinea-pigs as follows:

GUINEA-PIG NUMBER	WEIGHT	MATERIAL INJECTED	QUANTITY INJECTED	RESULT
	<i>grams</i>		<i>cc.</i>	
251	250	Serum rat 2	0.5	Died in 2½ days
249	250	(Same)	0.3	Died in 2½ days
215	290	Serum guinea-pig 2	2.0	Died in 2 days

Guinea-pig 2a weighing 280 grams was treated like guinea-pig 2 and 1 cc. of the serum, obtained as usual after seven and one-half hours, was injected into normal guinea-pig 252, weighing 290 grams. This animal died on the fourth day.

Protocol 3

Rat 3 (320 grams) receives 0.64 cc. and guinea-pigs 3, 4 and 5, weighing 270, 270 and 250 grams, receive respectively 0.54 cc., 0.54 cc. and 0.50 cc. of diphtheria toxin by intraperitoneal injection. Six and a half hours later all of the animals were bled and the sera obtained from the three guinea-pigs were pooled. The rat's serum and the pooled guinea-pigs' serum were injected into normal guinea-pigs as follows:

GUINEA-PIG NUMBER	WEIGHT	MATERIAL INJECTED	QUANTITY INJECTED	RESULT
	<i>grams</i>		<i>cc.</i>	
463	250	Serum of rat 3	0.8	Died in 3½ days
462	250	Serum of rat 3	0.5	Died in 4½ days
423	260	Serum of rat 3	0.5	Died in 3 days
427	About			
	250	Serum of rat 3	0.4	Died in 4 days
428	240	Serum of rat 3	0.35	Died in 14 days
429	260	Serum of rat 3	0.3	Lived
464	260	Pooled serum guinea-pigs 3, 4, 5	3.0	Died in 2½ days
426	260	Pooled serum guinea-pigs 3, 4, 5	2.0	Died in 4 days

Protocol 4

Rat 4 (170 grams) received 1.7 cc. of diphtheria toxin intraperitoneally and rats 5, 6 and 7, weighing 160 grams, 200 grams and 200 grams, received respectively 0.32 cc., 0.4 cc. and 0.4 cc. of toxin intraperitoneally. Between six and six and one-half hours later all of the animals were bled and the sera were obtained as usual. Serum 4 was kept separate; sera 5, 6 and 8 were pooled. These two sera were injected, subcutaneously into normal guinea-pigs as follows:

GUINEA-PIG NUMBER	WEIGHT	MATERIAL INJECTED	QUANTITY INJECTED	RESULT
	<i>grams</i>		<i>cc.</i>	
456	260	Serum rat 4 (1 : 10)	0.6	Survived
450	250	(Same)	0.7	Survived
452	250	(Same)	0.8	Died in 4½ days
322	260	(Same)	0.8	Died in 5½ days
324	255	(Same)	0.9	Died in 4 days
323	260	(Same)	1.0	Died in 2½ days
327	275	Serum rats 5, 6 and 7, undiluted	0.7	Died in 4½ days
316	250	(Same)	0.8	Died in 2 days
320	250	(Same)	1.0	Died in 2 days
458	250	(Same)	1.0	Died in 3 days

Protocol 5

Guinea-pig 317 (250 grams) received intraperitoneally 2.5 cc. of diphtheria toxin. Guinea-pigs 318, 319 and 321, each weighing 250 grams, received 0.5 cc. of diphtheria toxin each by intraperitoneal injection. Nearly seven hours later these animals were bled and the sera were obtained as usual. Serum 317 was kept separate; sera 318, 319 and 321 were pooled. The two sera were injected subcutaneously into normal guinea-pigs as follows:

GUINEA- PIG NUMBER	WEIGHT	MATERIAL INJECTED	QUAN- TITY INJECTED	RESULT
	<i>grams</i>		<i>cc.</i>	
452a	250	Serum guinea-pig 317	0.2	Died in 11 days
326	250	(Same)	0.3	Died in 4 days
37a	240	Serum guinea-pigs 318, 319 and 321	1.6	Died in 7 days
325	250	(Same)	1.8	Died in 3 days
321a	240	(Same)	2.0	Died in 3 days

In the preliminary experiment presented in protocol 1 it was found that six hours after the injection of 1 cc. of toxin per 100 grams the minimal lethal dose of the rat's serum was about 0.05 cc.; that of the guinea-pig's serum was about 1 cc. The results of more exact determinations of the toxicity of the sera under the conditions of this preliminary experiment are given in protocols 5 and 6. The lethal dose of the corresponding rat serum 4 was found to be 0.09 cc.; that of the corresponding guinea-pig's serum 317 was 0.3 cc. The difference in the toxicity of the two sera is decidedly greater in this experiment than it was in the first one.

Still greater differences in toxicity were observed when the quantity of toxin injected per 100 grams was less than 1 cc. For example, the lethal dose of the serum of rat 3 (which had received 0.2 cc. per 100 grams) was 0.4 cc., whereas that of the corresponding pooled guinea-pigs' sera 3, 4 and 5 was five times as much—2 cc.

The foregoing experiments seem to indicate that the cells of the rat are usually much less permeable to diphtheria toxin than are those of the guinea-pig. However, it is also conceivable that

the toxin does not actually permeate the cells of the susceptible guinea-pig, but merely attaches itself to a vulnerable element or "receptor" on the surface of the cells. The failure of this attachment to take place in the case of the resistant animal could account for the differences recorded in our experiments.

There is, thus, nothing in our results which can be considered as actually invalidating the side chain theory. All that can be asserted regarding the mechanism of the rat's immunity to the toxin is that the cells of this animal seem to possess the property of preventing the toxin from permeating them or of attaching itself to them.

SUMMARY

1. Zingher's method of injecting intracutaneously in guinea-pigs is described.

2. It is shown that constant results can be obtained with the Roemer technic only if the larger guinea-pigs are used (over 400 grams).

3. The rat is not absolutely immune to diphtheria toxin. Although it usually survives the injection of 1000 minimal lethal doses (for the guinea-pig) it regularly succumbs to 4000 such units.

4. The rat is capable of the production of antitoxin upon the repeated injection of diphtheria toxin.

5. The resistance of the rat to diphtheria toxin is not due to the presence of normal antitoxin, but to the property of the cells of preventing the toxin from entering them or of attaching itself to them.

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THE ACTION OF BACTERIAL CULTURE PRODUCTS ON PHAGOCYTOSIS¹

AUGUSTUS B. WADSWORTH AND E. N. HOPPE

From the Division of Laboratories and Research, New York State Department of Health, Albany

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Few of the bacterial poisons have been isolated or identified by chemical analysis; the true toxins are scarcely known save for the reactions they induce in animals. They can be recognized and their potency measured only when these reactions are characteristic as they are with tetanus, diphtheria and botulinus toxins. The febrile reactions and the development of immunity that are induced by other pathogenic species of bacteria in the course of many of the diseases of man suggest quite definitely the production of toxic substances capable of inciting specific immune reactions, but it has been impossible to detect or to measure accurately these substances.

This study was undertaken not only for the purpose of investigating further the action of the known bacterial poisons on the body cells, but also in the hope of finding some isolated animal cells or tissues sensitive to the unidentified bacterial substances, and a technic delicate enough to register degrees of injury to their normal reactions.

The first cells selected for this investigation—and the only ones dealt with as yet—were the leucocytes. These simple cells were chosen for the obvious reason that they were eminently suited for a study involving elementary life phenomena. They could be easily isolated; they would react to a variety of alterations of their environment, and those alterations could be quantitatively and qualitatively controlled.

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TECHNIC

A very simple technic was evolved after a long series of preliminary studies. Each operation was standardized. In this technic the phagocytic activity of the leucocyte for sensitized staphylococcus cells was selected as the index by which to evaluate the degree of the action of the test agents. In general, the method consisted of exposing well-washed leucocytes to the action of the substances to be tested. The effect of this exposure was then measured by the degree of phagocytosis that occurred when sensitized staphylococci were brought in contact with the leucocytes. This degree was determined from microscopical preparations, and stated in units as the phagocytic index.

The principle observed in developing the technic was that only one unknown factor should be present in each test and the elements of this unknown factor carefully controlled. The difficulties encountered in this process are exceedingly well pointed out and discussed by Fleming (1908) (1). The details of the finished technic are given here.

The materials used in the tests were: (1) a suspension of washed leucocytes, (2) an even emulsion of sensitized *Staphylococcus pyogenes aureus*, and (3) various toxins and culture broths which are collectively called test substances.

The leucocytes were obtained from the circulating blood of a normal dog by bleeding it with a syringe from the jugular vein. For each 1 cc. of leucocyte suspension required, 10 cc. of blood were taken. This blood was carefully mixed with an equal volume of 2 per cent sodium citrate solution to which 0.5 per cent sodium chloride had been added. It was immediately distributed in 15 cc. pointed centrifuge tubes, 10 cc. in each, where the white blood cells were collected in strata by centrifugalizing for thirty minutes at low speed, no. 3 on the rheostat (size 1, type B centrifuge, manufactured by the International Equipment Company). High speed centrifugalization mutilated the leucocytes. The leucocyte strata were removed from the tubes by the usual capillary pipette method and washed in ten times their volume of 0.85 per cent salt solution. The leucocytes were again separated by centrifugalizing and drawn off as before. The final suspension was then made in 0.85 per cent salt solution in the proportion stated above,

i.e., each 10 cc. of blood to contribute 1 cc. of leucocyte suspension. Many red cells were retained, but this was no disadvantage. Leucocytes obtained by aleuronat injections into the peritoneal cavity of small laboratory animals were quite unsuited because of their lack of uniformity in phagocytizing after being wholly or partially filled with aleuronat. Leucocytes from rabbits' blood were also rejected because of their extreme variation in number per cc. in different rabbits. There were no objections to leucocytes from horses' blood, but these were not as readily obtainable as dog leucocytes.

The staphylococci were grown eighteen hours on a beef infusion agar slant which was inoculated from a seed culture transferred every two or three days. The organisms from this eighteen hour culture were suspended in 8 cc. of 0.5 per cent salt solution and sensitized by adding 0.1 cc. of normal horse serum. This mixture was thoroughly agitated by repeatedly drawing it into and forcing it from a capillary pipette. It was then incubated at 37°C. for fifteen minutes. The organisms were quickly thrown down by centrifugalization. The supernatant fluid was discarded and replaced by 10 cc. of 0.5 per cent salt solution. The organisms were thoroughly separated and washed in this solution with a capillary pipette. After centrifugalization this washing was discarded, and the staphylococci were evenly suspended in 0.5 per cent salt solution to give an opacity equal to that of a no. 8 barium sulphate scale. A perfectly homogeneous suspension of the staphylococcus was more easily maintained in 0.5 per cent than in 0.85 per cent salt solution. Sensitization with normal horse serum was preferable to sensitization with staphylococcus immune rabbit serum because the organisms were more slowly and uniformly ingested, and beyond a certain point numbers of organisms in a leucocyte were not desirable. The number of staphylococci in the final suspension was given special attention—as was the case with the final leucocyte suspension—for at best this varied considerably. Controls were always included, but uniformity of results in the separate tests was desired in so far as it could be maintained, and the ratio of staphylococci to leucocytes was a factor.

The action of the culture material of different species under different conditions was tested with the leucocytes and staphylococci and constituted the variable factors in the experiments. The descriptive details of these are more logically and conveniently considered in connection with the tests in which they were used and are described in that connection.

The tests were performed *in vitro*.

Small test tubes, 1 cm. by 7 cm., were set up in racks and labeled. A tube was provided for each test substance and an additional tube for each factor that required a control. Then 0.1 cc. of each test substance or control substance was pipetted into its allotted tube, and 0.2 cc. of the leucocyte suspension was mixed with each. The racks of tubes were incubated at 37°C. for fifteen or thirty minutes for the test substances to act on the leucocytes. After this exposure, 0.1 cc. of the sensitized staphylococcus suspension was added to each tube, and the contents were mixed as before. The racks were returned to the incubator, and twenty-five minutes were allowed for phagocytosis to take place.

Smears were made from the phagocytic mixtures upon glass slides in the ordinary manner of blood smearing, and stained in Coplin jars with Wright's stain.

At least fifty polymorphonuclear leucocytes were counted and the following indexes determined: (1) the percentage of leucocytes phagocytizing, (2) the average number of staphylococci ingested by the phagocytes, and (3) the phagocytic index, which was the average number of staphylococci ingested by the fifty leucocytes, or by as many leucocytes as were counted.

Phagocytic technic *in vitro* has generally fallen into disuse on account of the extreme difficulty in securing reliable results with it. The numerous investigations and discussions of the purely technical phases of the problem will be omitted here. However, in working out the present technic it was obvious that variations in almost any factor could materially affect the final results. Even faulty technic in smearing a single slide could mutilate enough phagocytes to vitiate the results of an entire test. The possibilities for error were so numerous that none of this work was intrusted to assistants. Results were discarded that could not be readily duplicated. No conclusions were drawn unless the results of the experiment showed a definite and well-marked effect on phagocytosis.

EXPERIMENTS

In the first series of experiments the action of a variety of toxins and culture broths was tested on the leucocytes.

Standard diphtheria toxin was selected for the preliminary study, and it was found that after leucocytes had been exposed to its action for fifteen minutes they ingested almost no sensitized staphylococci as compared with the control preparations.

Then fifteen other toxins and culture broths of widely differing bacterial species were tested. The results were similar in every case to those obtained with diphtheria toxin. The extent of the inhibition of phagocytosis is well shown in the photomicrographs reproduced at the end of the paper.

In the table the toxins and culture broths and the controls are listed and described in the second column. The headings of the succeeding vertical columns follow the order of the steps in the established technic, and the last three columns give the resulting indexes.

From the phagocytic indexes of this table it is readily seen that the potency of the toxin as indicated by the minimal lethal dose did not affect phagocytosis.² Toxin no. 2 with a minimal lethal dose of at least 10 cc. was produced by an attenuated culture of the same strain that produced toxin no. 1 with a minimal lethal dose of 0.003 cc. The culture medium was similar in both cases. Yet, both toxins were equally depressing to the phagocytic index. Also, it may be seen that the hydrogen ion content was not a factor here; for example, the pH value of toxin no. 1 was identical with that of its control broth.

In the second series of experiments some facts regarding the nature of this depressing substance in toxins and culture broths were determined.

Diphtheria toxin was generally used, and the technic was the same as that for the first series. The tests readily fell into four groups: (1) the effect of the time of exposure, (2) the effect

² It is significant here to note the results of another study of the action of the bacterial toxins on the leucocyte (Wadsworth, A. B. and Vories, R.: The action of leucocytes and brain tissue on diphtheria and tetanus toxins. *Jour. Immunology*, 1921, 6, 413). In mixtures of diphtheria and tetanus toxin with leucocytes no loss in the potency of the toxin could be detected by either the minimal lethal dose or the intracutaneous test. Apparently there is no physical or chemical combination between these true toxins and the leucocytes, under the conditions of the experiment.

of neutralizing agents, (3) the effect of destructive agents, (4) the source of the depressing factor.

1. THE TIME OF EXPOSURE

Leucocytes were exposed to the action of diphtheria and tetanus toxins for varying lengths of time between the limits of two or three minutes and seven hours. Then staphylococci were added for phagocytosis. The degree of phagocytic depression, however, bore no relation to the time of the exposure of the leucocytes. The action of both of the toxins was immediate; and there was no evidence that the leucocytes later recovered in any degree by neutralizing the primary effect.

2. NEUTRALIZING AGENTS—ANTISERUMS

Diphtheria toxin-antitoxin mixtures were made with standard laboratory products as follows: (a) toxin with no antitoxin, (b) toxin with antitoxin to 1/100 neutralize, (c) toxin with antitoxin to 1/10 neutralize, (d) toxin with antitoxin to just neutralize, (e) toxin with antitoxin to several times neutralize, and, as usual, all the necessary controls—normal dog serum, normal horse serum, undiluted antitoxin, broth, salt solution—were included. These mixtures were left at 37°C. for varying lengths of time between the limits of two or three minutes and seven hours. Leucocytes were then exposed to the action of these test mixtures for fifteen minutes before the staphylococci were added. The resulting indexes showed that the antitoxin had no neutralizing effect on the depressing element in the toxin, nor did the toxin in small amounts act as a stimulant to the phagocytes.

Unconcentrated diphtheria antitoxic horse serum was allowed to act upon an equal volume of its homologous toxin for one hour. Leucocytes exposed to this test mixture for fifteen minutes gave as depressed indexes as the toxin control with no antiserum.

An agglutinating horse serum of high titer, produced by immunization with twenty-four hour cultures of diphtheria organisms washed free of all toxin, was used as a possible neutralizing agent for the phagocytic depressing substance in diphtheria

toxin of the homologous organism. The attempt was entirely unsuccessful. The agglutinating serum possessed no neutralizing elements.

3. DESTRUCTIVE AGENTS—LIGHT, HEAT, AND ENZYMES

Standard diphtheria and tetanus toxins were exposed to diffuse daylight for fourteen days and then to direct sunlight for three and one-half hours without in any way lessening the depressing effects of those toxins on the phagocytic action of the leucocytes.

The diphtheria, tetanus, and botulinus toxins and tuberculosis culture broth described in the table were subjected to varying degrees of heat. The upper limit was 120°C. for one hour under 15 pounds pressure in an autoclave. This heating in no instance produced the slightest effect upon the depressing element.

Dilute solutions of two proteolytic enzymes, trypsin and pepsin, in 0.85 per cent salt solution were allowed to act upon tetanus, diphtheria, and botulinus toxins and culture broths of the tubercle bacillus and of the staphylococcus for eight hours at 37°C. Leucocytes exposed to these digested bacterial toxins and culture broths as test substances for fifteen minutes before adding the staphylococci showed a much higher degree of phagocytic activity than the undigested toxin controls, and in some cases the depressing substance was completely destroyed. The only instance where no neutralization was apparent was that of trypsin in botulinus toxin. Possibly eight hours digestion was inadequate. Further work should be done on this point.

4. SOURCE OF THE DEPRESSING FACTOR—CULTURE MEDIUM

Diphtheria cultures of Park no. 8 organisms were grown in several experimental synthetic culture media for toxin production. The minimal lethal dose of the resulting toxins varied from 0.005 cc. to 0.1 cc. according as the constituents of the media were varied.³ When the action of these toxins was tested on leucocytes, it was found that the composition of the medium—which is most important in the production of standard diphtheria

³ These toxins were produced in this laboratory by Mary Wheeler in an investigation of the conditions affecting the production of the bacterial toxins.

toxin—in no way affected the production of the element which depressed the phagocytic index. All of the toxins were equally depressing.

The age of the cultures was found to be important in producing the depressing substance. This, however, varied with the organism; a three day culture broth of *B. Hofmanni* depressed the phagocytic index only 21 per cent, while that of staphylococcus caused a reduction of 55 per cent. The standard diphtheria toxin was cultivated seven days. The age of the toxins and culture broths used is included in the second column of the table.

The method of freeing the culture broths of organisms was immaterial—whether by centrifugalization or by Berkefeld filters.

This depressing substance has already been isolated from standard diphtheria toxin by adsorbing it to leucocytes and then lightly washing it from them with physiological salt solution. The leucocytes so treated regained their normal phagocytizing ability after the washing, the toxin used ceased to depress the phagocytic activity of normal leucocytes, and the salt solution washings took on the depressing property of the toxin for leucocytes in a phagocytic mixture.

SUMMARY OF EXPERIMENTAL RESULTS

To sum up the experimental section: First, the action of culture broths of thirteen widely differing pathogenic and saprophytic bacterial species was tested on phagocytes *in vitro*. In every case the phagocytic power of the leucocytes was inhibited in a high degree. Second, tests, chiefly with a standard diphtheria toxin, were done to determine some facts concerning the nature of this substance depressing to phagocytic activity and its relation to the true toxins. These tests showed that its action was immediate, and could not be neutralized by the ordinary anti-serums tested, nor destroyed by exposure to the degrees of heat or light used in the experiments. Variations in the constitution of the culture broths, which greatly affected true toxin production

caused no variation in the production of the depressing substance. The depressing action of young culture broths was found to be less marked than that of older cultures. It was also found that digestion with proteolytic enzymes either wholly or partially destroyed the depressing element. The substance could be isolated by adsorbing it to leucocytes and then washing it from them with salt solution. After removal of the substance the leucocytes regained their phagocytic activity.

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The action of toxins and culture broths in depressing the phagocytic power of leucocytes

	TOXINS, CULTURE BROTHS, AND CONTROLS	AMOUNT	DOC LEUCOCYTE SUSPENSION	EXPOSURE OF LEUCOCYTES TO TOXINS AND BROTHS AT 37°C.	SENSITIZED S. P. A.	INCUBATION AT 37°C.	AVERAGE NUMBER OF BACTERIA IN A PHAGOCYTE	PERCENTAGE OF PHAGOCYTING LEUCOCYTES	PHAGOCYTIC INDEX
		cc.	cc.	minutes	cc.	minutes			
1	Diphtheria toxin no. 161, * M.L.D. 0.003 cc., 7-day culture, pH 7.4.....	0.1	0.2	15	0.1	25	8.50	20	1.70
2	Control diphtheria toxin broth, pH 7.3.....	0.1	0.2	15	0.1	25	20.13	88	23.00
	Diphtheria toxin no. Um_2^+ , M.L.D. 10 cc. +, 7-day culture.....	0.1	0.2	15	0.1	25	5.40	20	1.08
	Control diphtheria toxin broth, pH 7.3.....	0.1	0.2	15	0.1	25	23.14	70	16.20
3	Tetanus toxin no. 93*, M.L.D., 0.0001 cc., 14-day culture.....	0.1	0.2	15	0.1	25	7.13	30	2.14
	Control tetanus toxin broth, pH 6.7.....	0.1	0.2	15	0.1	25	23.40	94	22.00
4	Botulinus toxin no. 1B, M.L.D., 0.005 cc., 10-day culture†, pH 6.2.....	0.1	0.2	15	0.1	25	9.34	46	4.30
	Control Van Ermengem broth, pH 7.5.....	0.1	0.2	15	0.1	25	28.55	76	21.70
5	Tuberculosis culture broth, virulent human strain no. 160, 21-day culture.....	0.1	0.2	15	0.1	25	14.23	26	3.70
	Control glycerin broth, pH 7.4.....	0.1	0.2	15	0.1	25	23.04	92	21.20
6	B. prodigiosus culture broth, 18-day culture, pH 7.8.....	0.1	0.2	30	0.1	25	12.14	14	1.70
7	Streptococcus culture broth, 18-day culture, pH 6.2.....	0.1	0.2	30	0.1	25	10.50	28	2.94

8	Pneumococcus culture broth, virulent strain, 10 day culture§	0.1	0.2	30	0.1	25	20.57	28	5.76
9	Pneumococcus culture broth, avirulent.....	0.1	0.2	30	0.1	25	8.33	6	0.50
10	Diphtheroid culture broth, 18-day culture, pH 8.2†	0.1	0.2	30	0.1	25	8.50	8	0.63
11	B. fecalis alkaligenes culture broth, 18-day culture, pH 7.8.....	0.1	0.2	30	0.1	25	5.75	8	0.46
12	Staphylococcus aureus culture broth, ** 18-day culture, pH 8.2.....	0.1	0.2	30	0.1	25	5.00	2	0.10
13	B. Hofmanni culture broth, 18-day culture, pH 7.8.....	0.1	0.2	30	0.1	25	15.00	6	0.90
14	B. coli culture broth, 18-day culture, pH 8.2.....	0.1	0.2	30	0.1	25	20.50	8	1.64
15	B. subtilis culture broth, 18-day culture, pH 8.2.....	0.1	0.2	30	0.1	25	15.71	14	2.20
16	B. diptheriae culture broth, Park no. 8 strain, *** 18-day culture, pH 8.2†	0.1	0.2	30	0.1	25	7.00	14	0.98
	Control broth, incubated 18 days, pH 7.45.....	0.1	0.2	30	0.1	25	16.40	64	10.50
	Control broth, not incubated, pH 7.45.....	0.1	0.2	30	0.1	25	14.45	74	10.70
	Control, 0.85 per cent salt solution.....	0.1	0.2	30	0.1	25	14.02	72	10.10

* Standard laboratory toxin.

† From an attenuated strain of Park no. 8.

‡ Toxin produced at room temperature.

§ These lines of investigation were originally started with the pneumococcus in 1907 but when they were resumed, attention was directed to the study of the better known toxins. The pneumococcus culture broth also has marked action on the red blood cells which is easily seen when it is added in excess; the mixture turns brown and the red cells may be laked. The leucocytes, however, persist and a few of them continue to be active phagocytes.

The pneumococcus cultures used in this protocol were originally the same Type I strain. The avirulent culture was obtained by attenuating the virulent strain in culture media (see "A study of the changes in virulence of the pneumococcus at different periods of growth and under different conditions of cultivation in media" by A. B. Wadsworth and M. B. Kirkbride, Jour. Exper. Med., 1918, 28, 791-805). The avirulent culture grew much more luxuriantly than the virulent culture but often in repeated tests the action of cultures of both strains on the leucocytes varied greatly.

** Same strain as that used to test phagocytosis in all tests.

*** Same strain as that used for standard toxin no. 161.

Note. Salt solution controls are not included since the results were always practically the same as those of the broth controls. The broth controls were in all cases from the same lot of broth as that in which the organisms were grown.

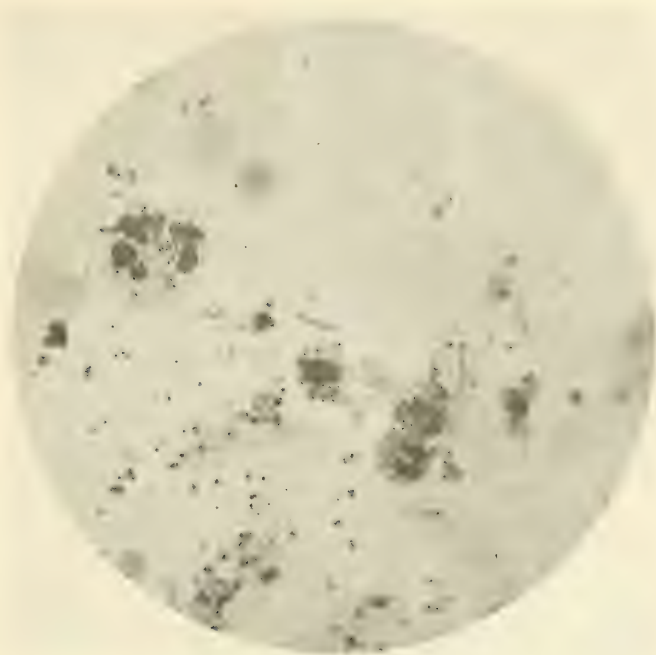


FIG. 1. PHOTOMICROGRAPH SHOWING NORMAL PHAGOCYTOSIS OF STAPHYLOCOCCI
IN A CONTROL PREPARATION

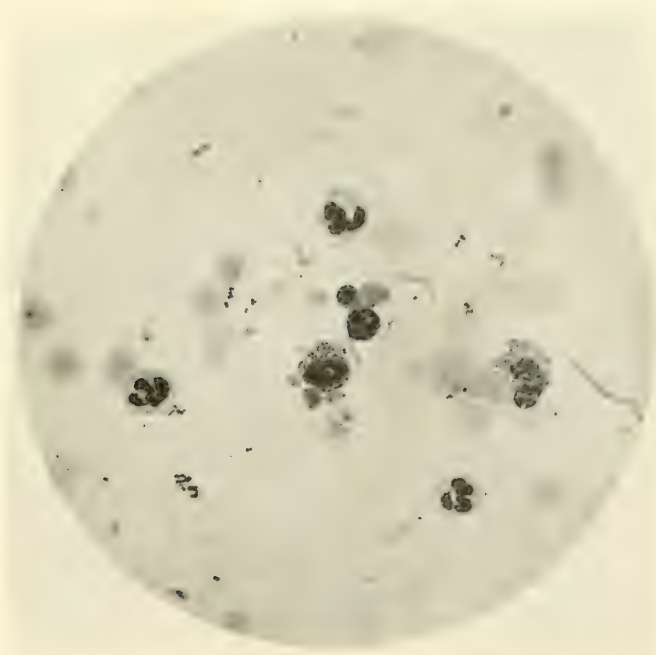
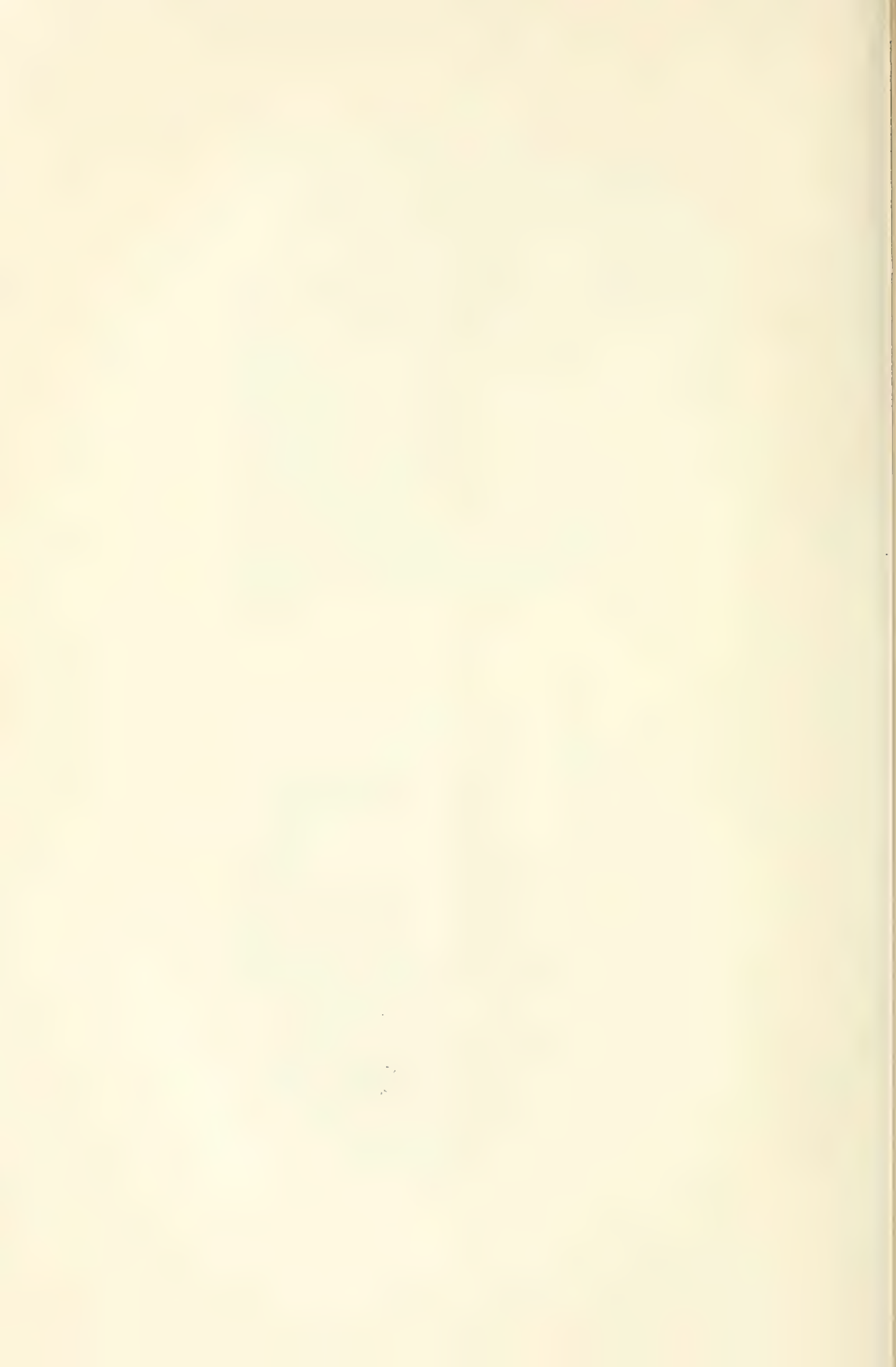


FIG. 2. PHOTOMICROGRAPH SHOWING THE INHIBITION OF PHAGOCYTOSIS OF
STAPHYLOCOCCI AFTER THE LEUCOCYTES HAD BEEN EXPOSED TO THE
ACTION OF A BACTERIAL CULTURE BROTH

None of the granulations in the eosinophyle near the center of the field is due to phagocytosis.



THE ACTION OF LEUCOCYTES AND BRAIN TISSUE ON DIPHTHERIA AND TETANUS TOXINS

AUGUSTUS B. WADSWORTH AND R. VORIES

*From the Division of Laboratories and Research, New York State Department of
Health, Albany*

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In any study of the action of the bacterial poisons on the leucocytes it is important to determine the chemical or physical reactions, if any, that take place between the leucocyte and the known bacterial toxins such as the diphtheria and tetanus toxins. Since the chemical or physical changes in these toxins are indicated and can only be measured by determining changes in the toxic effects they induce in susceptible animals, some experiments were done to detect any loss of toxicity in mixtures of diphtheria toxin and leucocytes such as Wassermann and Takaki (1) demonstrated with mixtures of tetanus toxin and brain tissue.

The results of these early studies, carried on in 1917, now have an additional significance since later experiments (2) have demonstrated the presence of substances in bacterial cultures which are adsorbed on the leucocyte and inhibit phagocytosis. These substances which depress the phagocytic activity of the leucocyte are apparently quite distinct from the true toxins and are not affected by the antitoxins or antiserums.

The experiments are divided into two series. In the first series the free toxin was measured by the intracutaneous test, that is, by dilution and the injection of 0.1 cc into the skin of a guinea-pig. The toxic action of the material was thus very accurately measured, much more so than by subcutaneous injection to determine the minimal lethal dose, the method that was used in the second series of experiments for purposes of comparison.

There were a few technical difficulties in securing leucocytes free from blood. With care, however, it was possible to inject

TABLE I
Tests to show antitoxic potency of leucocytes for diphtheria toxin

CELL SUSPENSION	AMOUNT OF CELL SUSPENSION USED	AMOUNT OF DIPHTHERIA TOXIN DILUTION USED	EXPOSURE OF CELL SUSPENSION TO TOXIN FOR NEUTRALIZATION†	METHOD OF INOCULATION OF GUINEA-PIGS	RESULTS
Guinea-pig leucocytes obtained from peritoneal cavity 24 hours after aleuronat injection. Leucocytes used without washing*	cc.				
	0.2	0.2 cc. dilution $\frac{1}{50}$ M. L.D. in 0.1 cc.	1 hour at 20°C.	0.1 cc. intracutaneously	Positive Schick reaction
	0.2	0.4 cc. dilution $\frac{1}{50}$ M. L.D. in 0.1 cc.	1 hour at 20°C.	0.1 cc. intracutaneously	Positive Schick reaction
	0.2	0.8 cc. dilution $\frac{1}{50}$ M. L.D. in 0.1 cc.	1 hour at 20°C.	0.1 cc. intracutaneously	Positive Schick reaction
Guinea-pig brain tissue suspension 0.1 gram to 1 cc. 0.85 per cent salt solution	0.2	2.0 cc. dilution $\frac{1}{50}$ M. L.D. heated	1 hour at 20°C.	0.1 cc. intracutaneously	No reaction
	0.2	0.2 cc. dilution $\frac{1}{50}$ M. L.D. in 0.1 cc.	1 hour at 20°C.	0.1 cc. intracutaneously	Positive Schick reaction
	0.2	0.4 cc. dilution $\frac{1}{50}$ M. L.D. in 0.1 cc.	1 hour at 20°C.	0.1 cc. intracutaneously	Positive Schick reaction
	0.2	0.8 cc. dilution $\frac{1}{50}$ M. L.D. in 0.1 c.c.	1 hour at 20°C.	0.1 cc. intracutaneously	Positive Schick reaction
	0.2	2.0 cc. dilution $\frac{1}{50}$ M. L.D. heated	1 hour at 20°C.	0.1 cc. intracutaneously	No reaction

* In other tests washed leucocytes were used.

† In other tests the exposures varied from one hour to twenty-four hours at temperatures from 5°C. to 20°C.

TABLE 2
Tests to show antitoxic potency of leucocytes for diphtheria toxin

CELL SUSPENSION	AMOUNT OF CELL SUSPENSION USED	AMOUNT OF DIPHTHERIA TOXIN DILUTION USED	EXPOSURE OF CELL SUSPENSION TO TOXIN FOR NEUTRALIZATION	METHOD OF INOCULATION OF GUINEA-PIGS	RESULTS TIME OF DEATH
Guinea-pig leucocytes obtained from peritoneal cavity 24 hours after aleuronat injection. Leucocytes unwashed	cc. 0.5	1.0 M.L.D. in 0.5 cc.	1 hour at 20°C.	1 cc. subcutaneously	Less than 64 hours
	0.5	2.0 M.L.D. in 0.5 cc.	1 hour at 20°C.	1 cc. subcutaneously	46 hours
	0.5	5.0 M.L.D. in 0.5 cc.	1 hour at 20°C.	1 cc. subcutaneously	Less than 39 hours
	0.5	1.0 M.L.D. in 0.5 cc.	1 hour at 20°C.	1 cc. subcutaneously	64 hours
Guinea-pig brain tissue suspension 0.1 gram to 1 cc. of 0.85 per cent salt solution	0.5	2.0 M.L.D. in 0.5 cc.	1 hour at 20°C.	1 cc. subcutaneously	46 hours
	0.5	5.0 M.L.D. in 0.5 cc.	1 hour at 20°C.	1 cc. subcutaneously	39 hours
	0.5	10.0 M.L.D. in 0.5 cc.	1 hour at 20°C.	1 cc. subcutaneously	23 hours
	0.5*	1.0 M.L.D. in 0.5 cc.	1 hour at 20°C.	1 cc. subcutaneously	Less than 39 hours

* Salt solution.

aleuronat into the pleural and peritoneal cavities of dogs and guinea-pigs without causing hemorrhage. Attempts to obtain large mononuclear lymphocytes in the exudate free from the polymorphonuclear leucocytes by waiting three or four days, failed. The exudate used in the tests was obtained twenty-four to twenty-eight hours after the injection of the aleuronat and contained chiefly the polymorphonuclear leucocytes. A heavy suspension of leucocytes was made in 0.85 per cent salt solution. They were tested both in their unwashed state and after they had been thoroughly washed in 0.85 per cent salt solution. A constant quantity, 0.2 cc. of the suspension of leucocytes was exposed in the dark to varying amounts, 0.2 cc., 0.4 cc., and 0.8 cc. of the standard diphtheria toxin so diluted that 0.1 cc. contained $\frac{1}{30}$ of a minimal lethal dose. The time allowed for neutralization was varied from one to twenty-four hours and the temperatures of the exposures from 5°C. to 20°C. Guinea-pigs were inoculated intracutaneously with doses of 0.1 cc.

For purposes of comparison an emulsion was made with 0.1 gram of the brain tissue of a guinea-pig in 1 cc. of 0.85 per cent salt solution. This emulsion was used instead of the suspension of leucocytes to determine the loss of potency in diphtheria and tetanus toxin. Controls with heated toxin were also inoculated.

A protocol of a typical experiment is given in table 1. In none of the experiments with fresh unheated toxin was there any neutralization of the toxin by the leucocytes. When the supernatant fluid from similar test-suspensions was injected after centrifugalization there was no loss of its toxic action in the skin. Furthermore, the washed leucocytes and the washed brain tissue sediments produced no reaction. In mixtures with diphtheria toxin, the leucocytes and the brain tissue reacted or failed to act quite similarly. There was apparently no combination of diphtheria toxin with either the leucocyte or brain tissue.

In the second series of tests a constant amount, 0.5 cc., of a heavy suspension of the guinea-pig leucocytes in 0.85 per cent salt solution was exposed to standard diphtheria toxin in dilutions varying so that doses of 1 cc. of the final mixtures would

contain 1, 2, 5, and 10 minimal lethal doses respectively. One hour at 20°C. was allowed for neutralization. Guinea-pigs were inoculated subcutaneously with 1 cc. doses of the mixtures. Controls consisted of similar preparations in which emulsions of the guinea-pig brain tissue were used instead of suspensions of leucocytes. A protocol of these experiments is given in table 2, and shows that the leucocytes had no diphtheria antitoxic potency.

When similar experiments were made with standard tetanus toxin 0.5 cc. of the guinea-pig brain emulsion neutralized at least 5 minimal lethal doses of the toxin. This result is in accord with the work of Wassermann and Takaki in 1898, but it has not been possible to find any previous observations to show that the leucocytes will not combine with either tetanus or diphtheria toxin or that brain tissue fails to combine with diphtheria toxin.

CONCLUSIONS

Neither the leucocytes of the dog, nor those of the guinea-pig, neutralize or combine with diphtheria or tetanus toxin. Although brain tissue combines with and neutralizes tetanus toxin, it has no action on diphtheria toxin.

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THE SPECIFIC ANTIGENIC PROPERTIES OF THE FOUR GROUPS OF HUMAN ERYTHROCYTES¹

SANFORD B. HOOKER AND LILLIAN M. ANDERSON

From the Evans Memorial, Boston

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I. THE MECHANISM OF HUMAN ISOHEMAGGLUTINATION

In view of the large number of investigations of the phenomenon of human isohemagglutination which have been made since its discovery twenty years ago, it is a curious fact that until within the last year no sustained attempt to elucidate the mechanism of the reaction has been made. Landsteiner's theory as to the number of agglutinins and agglutinable substances, their specific affinities and allocation, was so logically capable of extension to cover the fourth group later discovered by Decastello and Sturli (1902), and accounted for the observed facts so simply and adequately that it has been more generally acceptable than the explanations later offered by Hektoen (1907) and by Moss (1910). The prior and contemporaneous researches of Pfeiffer (1896), who had demonstrated the possibility of adsorption of bacterial agglutinins; of Ehrlich and Morganroth (1899), who showed that hemolysins were adsorbable; of Bordet (1899), who discovered that hemagglutinins could likewise be removed and that specific adsorption of bacterial agglutinins from a normal bivalent serum was brought about by contact with the respectively agglutinable organisms; and of Malkoff (1900), who demonstrated the same phenomenon with a normal trivalent hemagglutinating serum, all pointed very clearly to an appropriate method of attacking the problem of human isohemagglutination but until 1920 the theory lacked experimental support.

¹ Presented at the Eighth Annual Meeting of the American Association of Immunologists, Cleveland, Ohio, March 25, 1921.

Hektoen, on the basis of direct agglutination and adsorption experiments, recognized three main agglutinins and three corresponding agglutinable elements. His statement that either group II or III cells adsorb the agglutinin of group I serum for both groups II and III cells, we have been unable to confirm. The arrangement ascribed to Hektoen in table 1 does not appear in his publication but is our interpretation of his findings. It is not supported by recent investigations.

Moss concluded that there are at least three different isoagglutinins and three isoagglutininophilic receptors. He believed Landsteiner's explanation to be incorrect—"my results

TABLE 1

Theoretic allocation of human isohemagglutinins and agglutinable elements

GROUP	LANDSTEINER, V. DUNGERN		HEKTOEN		MOSS	
	Cells	Serum	Cells	Serum	Cells	Serum
I	0	ab	0	a	0	a
II	A	b	AC	b	AC	b
III	B	a	AB	c	AB	c
IV	AB	0			ABC	0

Capital letters designate antigenic complexes. Small letters designate corresponding agglutinins.

have led me to somewhat different conclusions"—but his published records are in no way incompatible with Landsteiner's theory. Moss offered two possible explanations. We have tabulated only the second of these, which he favored—but did not affirm—because he was able to show that group IV cells adsorbed from group I serum the agglutinins for groups II and III cells as well as those for group IV—a result quite at variance with his first proposed explanation.²

Landsteiner's theory accounts perfectly for all of the results obtained by Koeckert (1920) who used fractional adsorption of isoagglutinating sera and fractional saturation of isoagglutinable cells in studying the mechanism of the reaction. Unger (1920)

² The group numbers used in this report are those of Jansky's classification; discrepant numerical designations used by various authors have been transposed so to conform.

and Schütze (1921) also contribute exactly corroborative evidence obtained by specific adsorptions, although neither showed that groups II and III cells, used together or successively to adsorb group I serum, are able to remove the agglutinin for group IV cells—which is obviously an important point in determining the number of specific isohemagglutinins and agglutinable elements.

These three independent studies are completely in accord with the results we had obtained preliminary to our investigation of immune hetero-hemagglutinins the details of which, likewise confirmatory, are presented in section II of this communication.

Table 2, from our experiments, covers the adsorption tests essential to a demonstration of the applicability of Landsteiner's theory. There seems to be no reason for assuming the existence of more than two isohemagglutinins or two isoagglutinable complexes. Koeckert's fractional saturation tests seem particularly convincing, although the adsorption-dissociation-recombination experiment to show the presence of only two agglutinins in group I serum (p. 535-536) implies certain quantitative relationships to which there are frequent exceptions, and as reported, contains a mathematical fallacy. However, we feel sure, with respect to the individual bloods we have studied, that group IV cells contain all of the antigenic elements which groups II and III cells together possess and that the reverse is likewise true, in so far as isohemagglutination is concerned.

Do subgroups exist?

The possible existence of specific subgroups, "minor" agglutinins, etc., is still an open question. Langer (1903) claimed that one serum he examined contained no less than six kinds of isoagglutinins demonstrable by successive adsorptions with different agglutinable cells. He gave no protocol. von Dungern and Hirschfeld (1910c) assume that group II is made up of three subgroups, which they differentiated by means of certain group III sera. They also gave no quantitative details. Differ-

ent cells and sera often show very considerable quantitative differences in their inherent agglutinabilities and agglutinating potencies; these variables must be carefully controlled in order to avoid error in the type of experiment just cited; Hektoen likewise found interagglutination among certain members of group III and this cannot be explained by the fact that he classified III and IV individuals together in group III.

TABLE 2
Differential adsorption of human isohemagglutinins

SERUM	CELLS	ISOHEMAGGLUTININ TITER					
		Un-adsorbed	Adsorbed by cells of group				
			I	II	III	IV	II and III
I	I	0	0	0	0	0	0
	II	256	128	0	64	0	0
	III	128	128	64	0	0	0
	IV	128	128	64	64	0	0
II	I	0	0	0	0	0	
	II	0	0	0	0	0	
	III	32	32	32	0	0	
	IV	32	32	32	0	0	
III	I	0	0	0	0	0	
	II	256	128	0	128	0	
	III	0	0	0	0	0	
	IV	256	128	0	128	0	

The figures represent the weakest dilution of serum which showed definite agglutination when examined microscopically after one hour in water bath at 37°C.

Serum dilution, 10 parts; 5 per cent washed corpuscles, 1 part.

No allowance is made for the variable dilution of the original volume of serum caused by addition of the corpuscular sediment used for adsorbing.

Culpepper and Ableson (1921) think that the groups may "overlap," thus explaining reactions between members of the same group. Unger, in many thousands of tests, has occasionally found that when certain group II and group III bloods are tested against the bloods of other members of their respective groups slight agglutination habitually occurs; moreover sharp

reactions follow transfusion of such incompatible bloods. This unexpected clumping has also been observed with group I cells which have hitherto been considered to be inagglutinable by any normal human sera. In certain instances this inter-reaction was accounted for by the presence of that poorly understood phenomenon of autohemagglutination which is particularly likely to occur at room or lower temperature. The apparent failure, on the part of earlier investigators, to control this important factor weakens the force of their evidence as to the existence of "minor" isohemagglutinins.

Relative to blood transfusion

Although agglutination of certain group I cells by some sera, and of other cells by certain group-homologous sera, seems to occur but rarely and although Unger's observations have not been confirmed we endorse his recommendation that recipient and donor be tested directly against each other; it should not be assumed that bloods are compatible merely because typing with known groups II and III sera show that they belong in the same group. Furthermore, in the selection of donors, we wish to emphasize the necessity of determining compatibility by *both* the direct and indirect tests; we submit the following considerations in support of this recommendation.

1. The direct tests between recipient and prospective donors are necessary because of the likelihood of the existence of incompatible subgroups; these would not be detected if only indirect typing were done.

2. The indirect tests are necessary because certain patients have such weakly agglutinating sera that the donors' cells remain unclumped when tested directly by the slide method, yet serious reactions may follow transfusion in such cases (Karsner, 1921). Incompatibility under these conditions can be demonstrated by recourse to indirect typing with groups II and III sera.

In our adsorption experiments with normal and "immune" hemagglutinating sera we have used the slide method for pre-

liminary determination of the degree of agglutinin exhaustion. We have noted that such preparations, when examined microscopically after a thirty to sixty minute interval at room temperature would show no agglutination, but tubed mixtures of the same serum and cells after an hour in the water-bath at 37°C. very frequently exhibited definite clumping which often was apparent even without magnification. Quantitative relations were not responsible, so the greater delicacy of the tube method may be ascribed to more thorough admixture and to the higher temperature at which the reaction takes place. Beside the increased degree and rapidity of agglutination which occurs at the higher temperature, there is the advantage that autohemagglutination is less likely to cause confusion when tests are performed at 37°C. rather than at 20°C. In addition, the occurrence of hemolysis is more readily observed in tube than in slide preparations.

3. In any clinical procedure liable to be followed by "accidents," which, in the case of transfusion can be usually foreseen and prevented by proper compatibility tests, the laboratory examinations should cover all suspected possibilities and should be thoroughly controlled. When both direct and indirect tests are performed each serves as a check on the other and the liability of serious reactions due to group-incompatibilities is minimized.

Source of post-transfusion reactions

Although it seems certain that isohemagglutination is to a considerable degree a dependable index of incompatibility, it has not yet been conclusively demonstrated that those post-transfusion reactions which have been ascribed to group-incompatibility are actually due to agglutinative or lytic phenomena. One might suspect that possibly some constituent of blood plasma could be responsible for certain reactions. The following observations are of course only suggestive.

That the specific (antigenic) differences which permit a classification of human beings into four groups are not limited to reactions involving only the erythrocytes is evidenced by the

confirmed experience of Davis (1917) who found that the principles of blood grouping apply also to skin grafting. Todd and White (1910) refer to certain organ or tissue transplantations in which autoplasmic transplants are successful while homoio-plastic usually fail.

These differences, moreover, are not confined to cellular structures but are exhibited by the different specific normal hemagglutinins and lysins contained in different sera.

That the reactions due to incompatibility are not ascribable wholly to agglutination or lysis seems to be a logical inference from Karsner's observations to which reference has previously been made, and from the case which Thalhimer (1921) has just reported. In these cases the recipients belonged in a group that normally possesses agglutinins for the cells of the group of which the donors were members; this fact was determined by typing the bloods. But it so happened that these recipients possessed no demonstrable agglutinins for the donor's cells; they were thus adjudged to be compatible, but the transfusions were followed by severe reactions. It might be argued that although no agglutination was observed *in vitro* it may have occurred *intra vasam*. This possibility is a real one with respect to hemolysis, but it is remote in the case of agglutination. Ottenberg and Kaliski (1913) discuss this point at some length.

It was formerly the opinion that agglutination *per se* was not directly a source of danger except, of course, when massive—although it helps to defeat the purpose of transfusion by accelerating the removal of transfused cells from the circulation—but that hemolysis was the cause of severe reactions. Recent investigations tend to discredit this latter theory. Sellards and Minot (1916) found that hemoglobinuria can be produced in man by the injection of hemoglobin solution derived from laked human cells; rarely did subjective symptoms accompany the hemoglobinuria. This does not uphold the theory that post-transfusion reactions are due to lysis because in these cases subjective symptoms are much more common than hemoglobinuria.

Bowcock (1921) also concludes that severe reactions after repeated transfusions are anaphylactic manifestations and not due to hemolysis *per se*. Bayliss (1920), working with cats and dogs, found no evidence that any product of hemolysis is toxic and states his opinion that post-transfusion reactions are not caused by lysis of incompatible blood but "must be due to other concurrent phenomena," and ". . . are rather an aspect of the action of foreign serum protein analogous to that responsible for anaphylactic shock." This view, which we feel to be plausible, though probably not explanatory of all types of reaction, finds some circumstantial support in the conclusion of Bierry (1920) that species and individual characteristics are due to differences in the proteins of the blood plasma; in the two cases of serum sickness (urticaria) reported by Marie (1916) which occurred in typhoid patients after a second intravenous injection of *human* serum from typhoid convalescents, precipitins being demonstrable in each instance; in the occasional development of symptoms suggestive of serum disease—urticaria and other skin eruptions—which Ottenberg and Kaliski and others observed following their transfusions. Such reactions, however, apparently were correlatable neither with the presence nor absence of hemagglutinins; and the pertinence of Marie's observation—in its bearing on the question as to whether the index of actual incompatibility is afforded by isohemagglutination—is lessened because the group-relation of donors and recipients was not determined.

Meleney *et al.* (1917) also suggest that the "foreign protein" character of the donor's blood may be responsible for reactions because of (a) their absence when small amounts of blood are transfused, (b) their progressive increase with successive transfusions especially when the same donor is used, (c) the tendency of some donors to cause more reactions than others, (d) the occasional occurrence of a transient polynucleosis which is a feature of the reaction from intravenous injection of foreign protein; (e) they also noted the development of urticaria and localized areas of edema. In considering the possibility that post-transfusion reactions may be due to the alien-protein

nature of donor's plasma it should be borne in mind that extremely severe reactions would not be expected because the foreignness would naturally be of slight degree; perhaps so slight that differences could be demonstrated serologically only by the most delicate method known; that is, "immunization" within the same species.

The subject is complicated by many conflicting observations and opinions. Drinker and Brittingham (1919) report that the washed whole cell content of blood is uniformly toxic, whereas Sydenstricker, Mason, and Rivers (1917) maintain that washed group I cells can be given with impunity to members of any group, although occasionally "typical anaphylactic-like" reactions of mild grade may develop. It is of interest to note that these reactions are quickly relieved by adrenalin.

A further indication of the complexity of this question is afforded by the observations of von Dungern and Hirschfeld (1910b) who, by utilizing only a limited number of variously adsorbed animal sera, were able to differentiate at least twelve specific constituents in the erythrocytes of different humans; should these substances show variations independently, four thousand human bloods could be distinguished.

The last suggestion which we wish to bring forward in this connection is an anticipatory reference to an observation detailed in section II. Briefly, we found that the red cells of a group I blood contained a specific antigenic substance which was not present in the cells of those members of groups II, III, and IV that we examined. The bearing that this may have on the problem of transfusion can only be conjectured. If this particular individual's blood is fairly representative of group I—the "universal donor"—it would seem that the "foreign protein," which may be assumed to cause reactions, is not located in the erythrocytes because so many cases have received group I blood without notable reactions. That a specific isoagglutininogenic element is possessed by many members of group I is suggested by the observation that after transfusion of group I blood a considerable search is often necessary before inagglutinable bloods can be found for subsequent transfusions. Yet under

just these conditions—repeated transfusions of bloods that are adjudged compatible because no agglutination or lysis is found in preliminary tests—reactions occur with increasing frequency; and, as noted, this indicates an extracellular source of the reactions.

Absence of isoprecipitins in normal human sera

We have failed to find anything in the nature of an antigen-antibody reaction between normal sera alone that might throw light on the mechanism of reactions following *primary* transfusions. There was no precipitation or fixation of alexin when complete cross-tests were made, in adequately varied proportions, between normal sera from all four groups. The possibility that such isoantibodies may be formed *after* transfusion is indicated by Marie's observation but even such sequential development does not necessarily prove that post-transfusion accidents are mediated by an antigen-antibody reaction.

Summary

By means of appropriate adsorption tests it has been shown that Landsteiner's theory as to number and allocation of human isohemagglutinins and agglutinable elements is probably correct.

Evidence suggesting the possible existence of sub-groups is discussed, and other data are submitted in support of the recommendation that *both* the typing of bloods and direct tests between recipient and prospective donors be adopted as routine procedures preliminary to transfusion.

Attention is drawn to a number of observations which arouse suspicion, at least, that blood-plasma rather than cells may be the source of certain post-transfusion reactions. There is probably no etiologic or concurrent antigen-antibody plasma reaction following primary transfusions because we were unable to demonstrate any isoprecipitins or alexin-binding antibodies in cross-titrations of representative group sera.

Both from the clinical standpoint and because of the information which would derive to the subject of antigenic specificity,

serologic methods deserve more thorough application particularly to the problem of those reactions which follow repeated transfusions.

II. PRODUCTION OF GROUP-SPECIFIC ANTIHUMAN HETEROHEM- AGGLUTININS

An attempt to produce group-specific hemagglutinins in rabbits by repeated injections of human cells and adsorption of the resulting antisera has recently been made by Kolmer and Trist. Their antisera often showed somewhat higher titers for cells of the group used in immunization but adsorption to remove group antibodies resulted in complete exhaustion of agglutinins and lysins; they conclude that it does not seem possible to prepare group-specific immune sera. However, their procedure was to adsorb any given serum with mixed cells from the three other groups which were not used as inocula. A glance at table 1 shows that in each case all of the typical antigenic elements were used in every adsorption and the reason why all group-specific antibodies were removed becomes immediately apparent.

It has long been recognized that there must be, in any one group, an antigenic complex which is common to each of the other groups, because an immune agglutinative or lytic serum for any one group acts almost or equally as well on all the others. To the existence of this common antigen, Coca, in discussing the experiments just cited, attributed his own failure to demonstrate group-specific immune agglutinins by adsorption methods. Still, such "common" antigens exist in numerous bacterial families without offering any noteworthy obstacle to the differentiation of type strains by agglutinin-adsorption methods, so they should not, nor do they, prevent a similar differentiation of human erythrocyte groups. Coca used only one rabbit and, as will be seen, this accounts adequately for his failure.

von Dungern and Hirschfeld (1910 c) cite one germane experiment in which a dog and a rabbit were injected with group III cells. They developed agglutinins for all groups but acted most strongly on group III. Adsorption by group I cells

removed all agglutinins except those for group III. This experiment demonstrates the possibility of specific adsorption although in our experience differential exhaustion of a specific anti-group III serum by group I cells does not remove the agglutinins for either group III or IV. These authors (1908) were able also to elicit evidence of group-specific structures in dog's erythrocytes by investigating the properties of immune isohemagglutinins.

TABLE 3
Theory of production of group-specific sera

INJECTED WITH CELLS	AGGLUTININ DEVELOPED	ADSORBED BY GROUP	LEAVES AGGLUTININ	AGGLUTINATES GROUP	CORRESPONDS TO HUMAN GROUP
I—X	x	1 X	0	0	4
		2 AX	0	0	4
		3 BX	0	0	4
		4 ABX	0	0	4
II—AX	ax	1 X	a	2, 4	3
		2 AX	0	0	4
		3 BX	a	2, 4	3
		4 ABX	0	0	4
III—BX	bx	1 X	b	3, 4	2
		2 AX	b	3, 4	2
		3 BX	0	0	4
		4 ABX	0	0	4
IV—ABX	abx	1 X	ab	2, 3, 4	1
		2 AX	b	3, 4	2
		3 BX	a	2, 4	3
		4 ABX	0	0	4

Capital letters designate antigenic complexes. Small letters designate corresponding agglutinins.

An example of the astonishing degree of individual specificity demonstrable in a certain class of antigens is afforded by the observations of Todd and White (1910). They studied the specificity of the isolysins which were produced in oxen by injecting blood of other oxen. A mixture of 60 to 70 such isolytic sera adsorbed by the cells of one normal ox still hemolyzed the cells of 110 other cattle but had no action on the

individual's blood with which it had been adsorbed. Such an extreme degree of specificity would not be expected necessarily to develop when human blood is injected into rabbits; the sharpness, or delicacy of specific reactions becomes the more pronounced as the genetic relationship between the inoculated animal and the animal that furnishes the antigen becomes the closer. Thus, an iso-antibody would be the most refined differentiating reagent, and for the purpose of investigating the antigenic properties of human erythrocytes might perhaps be obtained from transfused cases; but inasmuch as there is some question as to the possibility of producing immune isohemagglutinins *de novo*, as our access to such cases has been limited, and as experimental conditions would unavoidably lack the necessary flexibility, we have confined our present study to the more conveniently obtained hetero-antibodies.

In the construction of table 3 we were guided by the results of our observations on the mechanism of human isohemagglutination and reckoned also with the presence of an antigen (X) common to each of the four groups. This table represents what might, *a priori*, be expected to result from appropriate immunization and adsorption experiments designed to detect group-specific immune heterohemagglutinins.

Technic

To simplify interpretation of results by reducing the number of variables we have used the cells of only one normal individual of each group for the production of immune sera. Healthy unused rabbits after a preliminary bleeding to obtain and examine their sera and cells, were injected intravenously with 1.0 ml of 50 per cent suspension of well washed cells on the first, second, and fifth days; on the eleventh day they were bled and their inactivated sera were tested for agglutinin content against cells of the four human groups. Subsequent injections were given usually on three successive days and bleeding was done five or six days later.

The volume of the progressively doubled serum dilutions used was ten times the dose of 5 per cent cell suspension. After one

hour in the water bath at 37°C. each tube was gently shaken to suspend the sedimented corpuscles and clumps, a loopful was quickly transferred to a glass slide and examined under low (60×) magnification. The figures in table 4 represent the weakest serum dilution in which distinct agglutination was demonstrable microscopically. In general these titers are from four to eight times higher than titers determinable with certainty by the naked eye. Adsorptions were done with washed corpuscular sediment; small doses were repeatedly added to serum, diluted 1:4 to 1:8, and from time to time the agglutinated cells were whirled out. When this primary serum dilution no longer showed—by the slide method—agglutination of the cells used for adsorbing, a set of dilutions was prepared, distributed into four series of tubes and tested against the four types of cells. A duplicate of the primary serum dilution was carried through under the same conditions except that no cells were added to it; this served to control the slight deterioration in antibody content which diluted serum undergoes at room temperature. The repeated addition of packed cells, which contain about 25 per cent of salt solution, caused a further moderate dilution of the primary serum preparation and often caused a fall in titer of one or two tubes. As we were chiefly concerned with qualitative data this factor was ignored except occasionally when we added to the control packed sheep cells in doses identical with the doses of human cells used in the actual adsorption test.

Normal rabbit-antihuman hemagglutinins

Prior to immunization the rabbit sera were tested for anti-human hemagglutinins; the results are recorded in the column headed "normal" in table 4.

It is apparent that all of the rabbit sera contained normal agglutinins effective in a dilution of 1:2 or higher for each type of cells. Five of the fourteen rabbits furnished sera that acted more strongly on cells of groups II and IV, which contain antigen "A." This selective property is specific as was readily shown by adsorption tests on serum 80; on the other hand, adsorption of 87 showed an absence of any group specificity. Moreover,

the agglutination of group III cells by 80 was specific; adsorption by group III cells did not remove agglutinins for II or IV, etc. Serum 80 behaved precisely like a human serum I.

The rabbits were so distributed among the four groups that we might observe whether the presence or absence of normal specific agglutinins had any effect upon the subsequent development of immune agglutinins. Apparently there was no consistent influence either upon potency or group specificity.

These rabbits possessed no demonstrable isoagglutinins.

Human antirabbit hemagglutinins

Each of the four types of human sera contained agglutinins for rabbit cells, active in dilutions ranging from 1:64 to 1:256 which, in general, corresponded with the isoagglutinin titers. There seemed to be no significant variation in the agglutinability of different individual rabbit's cells. Adsorption of each type serum by an individual rabbit's cells removed the agglutinins for all the other rabbits' cells; the presence or absence of group-specific antihuman agglutinins in the serum of the rabbit whose cells were used for adsorption was without influence on this result. This statement applies also to the following experiment.

Rabbit cells adsorb human isoagglutinin "b"

Human type sera from which we had removed antirabbit agglutinin were tested for isoagglutinins. It was found that "b" had been exhausted by the rabbit cells. Otherwise stated, group I serum still agglutinated II and IV cells but not III; groups II and IV sera were inactive; group III serum still agglutinated II and IV cells. von Dungern and Hirschfeld (1910 c) also observed that rabbit cells never adsorb isoagglutinin "a" but usually remove "b."

Insofar as our experiments extend, it thus appears that the cells of a given rabbit contain a complex which corresponds to human "B"—irrespective of whether or not that rabbit's serum contains normal group-specific agglutinins for human cells.

Were this "b"-adsorbing constituent of rabbit-cells antigenically identical with human "B" one would expect, because of "*horror autotoxicus*," that the injection of group III or IV cells would fail to produce a group-specific "b" agglutinin. This is not the case; both 80 and 85 whose cells adsorb human "b" proved capable of producing group-specific "b" agglutinin as shown in table IV. Possibly this "b" adsorbing substance belongs among the heterophil (Forssmann) "antigens;" we have not examined its nature further.

Group-specific immune heterohemagglutinins

Our data on immune rabbit antihuman hemagglutinins are collected in table 4. The positive results obtained particularly with animals 81, 77, 80, and 85 are for the most part in direct and striking confirmation of the accepted explanation of the mechanism of human isohemagglutination. It is evident that by appropriate adsorptions of these immune sera one can duplicate the specific isoagglutinative properties of any type of human serum. For instance, adsorption of an anti-group IV serum with group I cells furnishes a serum which still agglutinates groups II, III, and IV cells as does human group I serum; adsorption with group II cells produces a duplicate of human group II serum—cells of groups III and IV are agglutinated while I and II cells are unaffected; group III cells remove agglutinin for I and III, group IV cells entirely exhaust the serum of antihuman agglutinins, and a mixture of II and III cells does likewise. Incidentally, we may mention that using artificial duplicates of II and III sera, we have examined 250 specimens of human blood; in every instance the reaction has been the same as that obtained with known group II and III human sera.

Comparison of the expected with the actual results (tables 3 and 4) reveals only two discrepancies. The first—not wholly unexpected—is that heterologous adsorption of anti-group I serum, 81, does not remove the agglutinins for group I cells. Therefore these group I cells possess an antigenic complex which is not present in any of the other groups. This explains the

TABLE 4
Production of group-specific heterohemagglutinins

Production of group-specific heterologous sera							
RABBIT NUMBER	AGGLUTI- NATE CELLS	AGGLUTINATION TITRE					
		Normal	Immune unadsorbed	After adsorption by			
				I	II	III	IV
Antisera for group I cells							
81	I	2	512	2	512	512	256
	II	2	256	2	4	8	<2
	III	2	256	<2	4	4	<2
	IV	2	256	<2	4	4	<2
91	I	2	256	8	64	64	128
	II	2	512	8	<8	16	<8
	III	2	256	8	<8	8	<8
	IV	2	256	8	<8	<8	<8
94	I	4	8192	16	32	16	32
	II	16	4096	16	<8	<8	8
	III	4	4096	16	<8	<8	8
	IV	8	4096	16	<8	<8	8
Antisera for group II cells							
77	I	2	256	<8	8	8	<8
	II	2	512	512	8	512	<8
	III	2	256	8	8	<8	<8
	IV	2	512	512	8	256	<8
79	I	2	512	<8	<8	<8	8
	II	2	512	<8	<8	<8	<8
	III	2	256	<8	<8	<8	8
	IV	2	1024	<8	<8	<8	8
84	I	2	256	<8	<8	<8	<8
	II	32	512	16	<8	16	<8
	III	2	256	<8	<8	<8	<8
	IV	16	256	<8	<8	<8	<8
87	I	2	64	<2	<2	<2	<2
	II	2	128	2	<2	<2	<2
	III	2	32	<2	<2	<2	<2
	IV	2	16	<2	<2	<2	<2
93	I	16	256	<8	<8	<8	8
	II	8	256	<8	<8	<8	<8
	III	8	128	<8	<8	<8	<8
	IV	4	512	<8	<8	<8	<8

TABLE 4—Continued

RABBIT NUMBER	AGGLUTI- NATE CELLS	AGGLUTINATION TITRE					
		Normal	Immune unadsorbed	After adsorption by			
				I	II	III	IV
Antisera for group III cells							
80	I	2	256	<4	<4	<4	<4
	II	16	256	256	<4	8	<4
	III	8	2048	2048	1024	<4	<4
	IV	16	2048	2048	1024	4	<4
82	I	2	512	16	16	16	8
	II	2	512	8	8	16	<8
	III	2	512	8	<8	8	<8
	IV	2	512	<8	<8	8	8
92	I	16	4096	8	16	16	32
	II	16	4096	16	<8	8	16
	III	8	4096	16	16	8	8
	IV	2	4096	16	16	<8	16
Antisera for group IV cells							
83	I	2	256	<8	<8	8	<4
	II	2	256	<8	<8	8	<4
	III	2	256	8	8	<8	<4
	IV	2	128	<8	<8	<8	<4
85	I	2	2048	<4	<4	4	<4
	II	8	4096	4096	<4	4096	<4
	III	2	2048	1024	1024	4	<4
	IV	8	4096	2048	2048	2048	<4
90	I	2	512	<8	<8	<8	<8
	II	16	2048	2048	8	2048	16
	III	4	512	256	8	<8	<8
	IV	8	2048	2048	16	2048	16

rather curious fact that the only absolutely group-univalent serum obtained was for I cells, which normally are not isoagglutinable. The bearing which the existence of this unique antigen may have on the problem of transfusion reactions has been discussed in section I.

The second case of disagreement between observation and theory was disclosed by examination of serum 80. Adsorption

by group I cells failed to remove agglutinins for II cells. Repeated tests showed consistently the same result. We have considered a number of possible explanations of this discrepancy but none of them seems even to approach adequacy. Collateral evidence fails to uphold any technical error, or the possible existence of an unsuspected antigenic complex common to group II and III cells, as a cause of the anomaly. Is it the individuality of the animal?

Serum 90 is also peculiar in that II cells remove all agglutinin. This is likewise, to us, inexplicable.

Hemolysins

Although we have not made complete quantitative studies on specific adsorption of immune hemolysins or alexin-fixing antibodies, all the requisite qualitative tests revealed an exact parallelism with the results—including discrepancies—of agglutinin adsorption. As antigen in our fixation experiments we used suspensions of human red-cell stromata prepared by Vedder's (1919) method. Considerable economy of materials and more constant experimental conditions can be conveniently attained by this method than by direct titration of hemolysin. Both methods are somewhat complicated by the fact that many guinea-pigs' sera, used as alexin, contain a hemolysin specifically active against group II and IV cells, as Williams (1920) has shown, so preliminary adsorption is often necessary.

Influence of animal's individuality

Table 4 shows that in the production of group-specific antibodies in different rabbits we have failed about twice as often as we have succeeded. By injection of group I cells there was obtained one group-specific serum (81) one of less pronounced specificity (91) and one practically non-specific (94). This group-specificity entirely disappeared after the second and third series of injections, but it has remained unchanged for over four months in the immune serum obtained from the first bleeding of 81.

Using group II cells group-specific serum was furnished by only one of five rabbits. This specificity was not impaired by subsequent injections and has remained unchanged for over a year.

With III and IV cells group-specificity was demonstrable in each case in only one of three rabbits. Further series of injections likewise had no effect on the qualitative reactions, which have remained constant in sera stored for several months.

What are the causes of such irregularities in the specificity of antibody response?

Degree of immunization

In our method of preparing these antisera we were led to use few doses of rather small size prior to the first bleeding and then to follow closely the effect of subsequent injections, on account of the observation of Magnus (1908) who maintained that the degree of immunization determines the range of reaction. Working with cereal proteins he found that increased polyvalency of his precipitating antisera developed as the number of injections was increased. Lake, Osborne, and Wells (1914) confirmed and extended this observation by showing that after a few injections of hordein or gliadin the resulting antisera showed specific alexin-fixation with the homologous protein but after a further series of injections fixation was obtained with (related) heterologous proteins. Precipitin and passive anaphylaxis experiments gave similar results. Occasionally in the literature there are incidental records of analogous results with bacterial antigens. Butterfield and Neill (1920), for example, mention that more nonspecific agglutinins develop on "further injection of rabbits" with type strains of meningococci.

With regard to erythrocytes, Friedberger and Dorner (1905) claim that small doses produce a serum of higher specificity than do large doses, as determined by tests against cells of other animal species.

It may be presumed that the progressive development of polyvalency during the course of immunization is due to the presence, in the inoculum, of a mixture of proteins; or, in the

case of a "pure" protein, of multiple molecular groupings which possess distinctive antigenic properties. This hypothesis further presupposes that the various antigenic substances are present in widely different proportions. The predominating substance would lead, early in the course of immunization, to the production of a considerable amount of specific homologous antibody while other admixed antigenic elements, present only in minimal quantities, would produce appreciable amounts of antibody only after a number of injections.

Working, as we have, with "strains" of cells belonging to individuals of the same race, afforded an opportunity of applying this reasoning to a determination of whether human erythrocytes contain mixtures of the same antigens, in different proportion, or whether certain types contain certain antigens exclusively. Briefly we may say that the latter condition seems to be the actual one; we observed no evidence which would warrant any suspicion that antigens "A" or "B" are present in small amounts in group I cells, or that the other groups contain any of the newly discovered specific antigenic component that we demonstrated in group I cells. It is understood, of course, that this statement applies only to the individual bloods which we studied, and is further limited by our range of doses—totaling 10 in number and about 4.0 cc. of corpuscular sediment in amount.

We are unable to make any positive statement regarding the cause of individual variation among rabbits in their specific response to human erythrocytes. Our data are comparatively few and from them we have so far failed to establish any significant correlation between this variation and the presence or absence of normal hemagglutinins or hemagglutininogens; or the number of injections; or the titer or age of the serum; or the type of blood cells injected; or the age, breed, or sex of the animal.

However, these experiences illustrate very forcefully the great importance of taking into account the individuality of the animals used in studies of antigenic specificity. It has long been recognized that animals of *different* species produce immune sera that vary widely in valency. A horse, for example,

may furnish a strikingly multivalent serum by direct agglutination test, even when injected with a single type strain of meningococcus, whereas rabbits commonly furnish type specific agglutinins for this organism. Undoubtedly the number of injections is an additional important factor in this case.

Of particularly absorbing interest also are the studies of Bull and McKee (1921) on the antipneumococcus protective substances in normal chicken serum. Serologic classification in terms of these substances allocates types IIa and IIb in two distinct main groups while Type II strains compose a subgroup to both of them—the reverse of the common grouping by means of immune rabbit or horse sera.

It is a familiar fact that animals of the *same* species differ markedly in the *degree* of their response to a given antigen, but the variations in *specific* response have not been stressed in proportion to their fundamental biological importance.

As far as our work with erythrocytes is concerned one might suspect that the phenomena of "organ-specificity" would have some bearing on these variations. We were injecting antigenic cells practically identical in morphology and function with cells that the rabbit itself possessed. The analogy with lens substance reactions immediately presents itself. But it should be borne in mind that although both kinds of erythrocytes fulfil the very highly specialized function of oxygen-carriers, really this office belongs to the non-antigenic hemoglobin. There is no evidence that human and rabbit stromata contain any truly antigenic principles in common, although most rabbits' cells parallel the specific behavior of human group III cells in adsorbing isoagglutinin "b."

Furthermore, there are a few reports of similar variations in specific response to other blood constituents, *and* to bacterial antigens, so it is very unlikely that the explanation is to be found in any degree of community of antigenic components between the substance injected and the constitutional make-up of the animal receiving the injection. Johnson (1916) observed that of two rabbits receiving parallel injections of beef serum, one responded with the production of a specific hemolytic and

precipitating serum while the other rabbit's serum was specifically hemolytic but devoid of precipitin. The antigenic factor was the constant, the disparity of response must be attributed to the individuality of the antibody producing mechanism. Brown-ing and Wilson (1920) found that only certain individual rabbits produce alexin-fixing antibodies to globin. Evans (1920) working with meningococci, reports that sometimes several rabbits had to be injected before a tropin-containing serum could be procured, "due presumably to the peculiarities of different rabbits." A more pertinent observation was made by Butterfield and Neill. They obtained three antisera from different rabbits for each of fourteen representative strains of meningococci. Of these fourteen sets, four only showed the same agglutinative specificity when tested against the other strains. In six other sets each serum differed from the other two, and in the remaining four sets, two of the sera were alike and the third was at variance. These differences occurred mostly in the non-specific agglutinins, but with six of the sera, representing five strains, adsorption tests conspicuously revealed the actual individual diversity of specific antibody response to a given antigen. The authors did not comment on these observations; their bearing on the validity of many serologic classifications is obvious.

The comparative paucity of data precludes further discussion, but we wish to emphasize the fact that in studies of antigenic specificity or of any phenomenon analogous thereto, the individuality of the injected animal must be regarded not as a constant but as a possible variable of great importance.

Summary

Normal rabbit sera possess weak agglutinins for the four groups of human erythrocytes. In certain rabbits this agglutinative capacity is group-specific, being particularly marked for groups II and IV cells.

Each of the four types of human sera contains agglutinins for rabbit erythrocytes. No group-specificity is manifested.

Human isohemagglutinin "b," present in group I and II sera, is specifically adsorbed by rabbit cells.

Group-specific hemagglutinins, demonstrable by appropriate adsorption, were produced by injection of rabbits with type cells. The specific isoagglutinative activity of any type of human serum can thus be duplicated.

Group I cells, at least those of certain individuals, were found to contain a specific agglutininogenic component that does not exist in the other group cells examined.

Group-specific hemolysins or alexin-binding antibodies are developed with the agglutinins.

Many rabbits fail to produce group-specific antibodies; no cause for this individual variation was discovered. The fundamental importance of the animal's individuality in its bearing on serologic studies is emphasized.

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THE INFLUENCE OF TEMPERATURE UPON THE AGGLUTINATION OF THE RED BLOOD CORPUSCLES

FREDRIK JERVELL

From Rikshospitalet, Kristiania, Norway

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The agglutination test of red blood corpuscles is made either macroscopically or microscopically.

In the microscopic test the agglutination, as a rule, is manifest after a few minutes at room temperature.

In the macroscopic test, however, the agglutination is distinct only after a longer time. This test, which is often combined with a hemolytic one, is ordinarily made by adding 0.5 cc. of a 2 to 5 per cent suspension of washed red blood corpuscles to 0.5 cc. of serum. This mixture is left in an incubator for two hours and then in the ice chest over the night, when the reaction is read.

There is no doubt that the hemolysis occurs more quickly at 37° than at a lower temperature, and it seems to be a common opinion that the same is true with respect to the agglutination of the corpuscles.

As far as it may be seen, however, no experiments have been made to decide this question. It has, so to say, been considered an axiom that the agglutination should take place more quickly at 37° than at a lower temperature, and agglutination tests—with bacteria as well as red blood corpuscles—are as a rule made at a high temperature (37°–55°).

During some work on the isoagglutination of red blood corpuscles in man, the writer in a series of experiments customarily left the tests one to two hours at 37° and then over night in the ice chest. Some of the tests, however, were put directly into the ice chest without having been in the incubator. In com-

paring these tests with the first mentioned it was evident that no distinct difference was found in the degree of agglutination in tests made after either of the two methods.

Some tests were made, therefore, in order to find out at which temperature the agglutination of the corpuscles is strongest—8° or 37°.

EXPERIMENT I

To 0.5 cc. of an agglutinating and hemolytic serum from one individual were added 0.5 cc. of a 1 per cent suspension of washed red blood corpuscles from another individual.

Two tests were made. One was put into the incubator, the other into the ice chest. The reaction was read after one hour.

Table 1 shows a better agglutination in the ice chest than in the incubator. The hemolysis, however, occurred only in the incubator.

TABLE I
After one hour

	IN INCUBATOR	IN ICE CHEST
Agglutination.....	+	++
Hemolysis.....	+	—

Similar tests have been carried out with serum not showing hemolysis, but only agglutination and with corpuscle suspensions of different concentrations.

In these tests the amount of corpuscles used is of considerable importance. If the concentration of the suspension is too great and especially if the titer of the serum is a low one, the unagglutinated corpuscles will interfere with the reading of the result.

In all experiments where a low concentration of corpuscles was used—0.25 to 0.50 per cent—a better agglutination was obtained at 8° than at 37°. This was occasionally controlled by counting of the unagglutinated corpuscles. The number of these was always larger in tests carried out at a high temperature.

In order to find out whether the influence of temperature is the same in tests with different salt concentrations, which—as

is well known—is of great importance for the agglutination, the following experiments were made.

EXPERIMENT II

Two tests were set up with an agglutinating serum of high titer (1:80) of which only 0.1 cc. was used in each tube. In this way the salt function of the serum was eliminated as much as possible. 0.05 cc. of a 5 per cent suspension of blood corpuscles were measured out into each tube. Sodium chloride solution (0.5–5 per cent) up to 1 cc. fluid was then added.

Thus: Serum 0.1 cc. in 1 cc. fluid (dilution 1:10). Blood corpuscles 0.05 cc. of a 5 per cent suspension in 1 cc. fluid (concentration 0.25 per cent). Salt concentration 0.5 per cent, 1 per cent, 1.5 per cent, 2.0 per cent and so on.

The first series of tubes was put in the incubator, the second in ice chest and both series were examined after one and one-half and twelve hours.

It is shown in table 2 that the agglutination was more pronounced at 8° than at 37°, and that it was strongest at a salt concentration of 1.0 to 2.5 per cent.

TABLE 2

DILUTION OF SERUM	SUSPENSION OF CORPUSCLES	CONCENTRATION OF SODIUM CHLORIDE	AGGLUTINATION			
			Ice chest (8°)		Incubator (37°)	
			1½ hours	12 hours	1½ hours	12 hours
	<i>per cent</i>	<i>per cent</i>				
1:10	0.25	0.5	+	+	tr.	tr.
1:10	0.25	1.0	++	++	±	±
1:10	0.25	1.5	+++	+++	++	++
1:10	0.25	2.0	++	+++	++	++
1:10	0.25	2.5	+	+++	+	+
1:10	0.25	3.0	—	+	+	+
1:10	0.25	3.5	—	±	—	tr.
1:10	0.25	4.0	—	±	—	tr.
1:10	0.25	4.5	—	±	—	tr.
1:10	0.25	5.0	—	tr.	—	±

± = slight agglutination

tr. = trace.

The reason why the agglutination is more pronounced at low temperature may be that there is a better or quicker adsorption of the agglutinin at the low temperature than at the high temperature.

Some experiments were made to give information on this point.

With a serum of a known agglutinin titer adsorption tests were carried out at 8° and at 37°, the same corpuscle concentration being used. The attempt was then made to find some difference in the titer of the supernatant fluids after adsorption at the two different temperatures. In both tests, however, the amount of agglutinin was too small to allow the observance of a distinct difference. Another method therefore had to be used.

EXPERIMENT III

The purpose of the experiment was to find the amount of blood corpuscles required to adsorb a certain amount of agglutinin at different temperatures.

Four tests are set up. In each test were used 0.5 cc. of a highly agglutinating serum—not hemolytic—and 0.5 cc. of blood corpuscles. The latter had been prepared in varying concentration by diluting 0.05 cc., 0.1 cc., 0.15 cc., 0.2 cc. et cetera of a 50 per cent suspension up to a total volume of 1 cc. The ultimate concentrations of the corpuscles in the test mixtures were thus 2.5 per cent, 5 per cent, 7.5 per cent, 10 per cent, and so on.

Of the four series of tubes, all of which were closed with rubber-stoppers, the first series was put into the ice chest (8°), the second was left at room temperature (18°), the third was put into a water bath (25°), and the fourth into the incubator (37°).

After half an hour all tubes were centrifuged. With a Wright capillary pipet a minimal amount of serum was drawn from each tube, and a microscopic agglutination test was carried out to decide in what tubes agglutinin was still present in the supernatant fluid.

It is shown in table 3 that a considerably larger amount of blood corpuscles is required to adsorb the agglutinin at a high

than at a low temperature. The reason for this may be an easier or a quicker adsorption at the low than at the high temperature. The same was found in experiments made at higher temperature (45°-55°) where a still greater concentration of corpuscles was required to adsorb all the agglutini.

TABLE 3

SERUM	SUSPENSION OF CORPUSCLES	AGGLUTINATION BY SUPERNATANT FLUID AFTER ADSORPTION ONE-HALF HOUR			
<i>cc.</i>	<i>per cent</i>				
0.5	2.5	+	+	+	+
0.5	5.0	tr.	+	+	+
0.5	7.5	-	+	+	+
0.5	10.0	-	+	+	+
0.5	12.5	-	+	+	+
0.5	15.0		tr.	+	+
0.5	17.5		-	tr.	+
0.5	20.0		-	-	+
0.5	22.5		-	-	tr.
0.5	25.0		-	-	-

tr. = trace.

As a control on the results obtained a new experiment with the same serum was set up. The two experiments gave identical results.

It was further found that a concentration of corpuscles that was incapable of adsorbing all agglutinin at a high temperature, was capable of doing so when the mixture, after being shaken was placed at a lower temperature. In control tubes that stayed the same time at high temperature the agglutinin was not adsorbed.

After the corpuscles, as shown above, have adsorbed a larger amount of agglutinin at low temperature, it was thought that these corpuscles would lose some of agglutinin when exposed to a higher temperature. To test this hypothesis the following experiment was made:

EXPERIMENT IV

In a series of tubes 0.5 cc. of agglutinating serum were mixed with 0.1 cc. of a 50 per cent suspension of corpuscles. All the tubes were placed at 8° for one-half hour, during which time the corpuscles were agglutinated and settled out. The clear fluid microscopically examined for any agglutinating power (as in experiment III). All agglutinin was found to have been adsorbed. The tubes were then placed at 8°, 18°, 25°, 37°, 45°, 55° and 62° and after one-half hour they were centrifugalized and the supernatant fluid was examined for agglutinin.

The table shows that the corpuscles, after having adsorbed all of the agglutinin at a low temperature, lose part of it when brought into a higher temperature, but retain it all as long as they remain at the temperature in which the adsorption was made.

TABLE 4

	AFTER ONE-HALF HOUR AT						
	8°	18°	25°	37°	45°	55°	62°
Agglutination by supernatant fluid.....	—	—	tr.	+	++	++	+

tr. = trace.

EXPERIMENT V

The previous experiment was repeated with corpuscles that had adsorbed the greatest possible amount of agglutinin at 8° and had been washed afterwards in cold saline (4°) until all agglutinin had disappeared from the supernatant fluid. The corpuscles were then emulsified in cold saline (10 per cent suspension) and distributed in 7 tubes with 1 cc. in each. These tubes are placed at 8°, 18°, 25°, 37°, 45°, 55° and 62° for one-half hour. Before the tubes were put away the suspension fluid was examined and found to contain no agglutinin.

The table shows the same result as the previous experiment. The agglutination is less pronounced at 62° than at 45° and 55°

evidently because of a partial destruction of agglutinin at this temperature.

TABLE 5

	AFTER ONE-HALF HOUR AT						
	8°	18	25°	37°	45°	55°	62°
Agglutination by the supernatant fluids.....	—	—	±	+	++	++	+

± = slight agglutination.

SUMMARY

The experiments show a marked difference in the agglutination of red corpuscles at low and at high temperatures.

The most pronounced agglutination is obtained in the ice chest. It is shown that this is due to a quicker or more nearly complete adsorption of agglutinin at low than at high temperature.

After adsorption at 8° the corpuscles again lose part of the agglutinin when brought into higher temperatures.

The experiments seem to indicate that the maximal adsorption of agglutinin is different at different temperatures and more nearly complete at the low than at the high temperatures. When, therefore, the adsorption has been carried out at a low temperature and the corpuscles after that are placed at a higher temperature, they can retain only the quantity of agglutinin that corresponds to the maximum for the respective temperature and accordingly lose agglutinin until this maximum is reached.

CONCLUSION

1. The red blood corpuscles show a more pronounced agglutination at low than at high temperatures when tested with isoagglutinins.

2. The corpuscles adsorb in a given time a larger amount of isoagglutinins at a low than at a higher temperature.

3. Agglutinin adsorbed at a low temperature (8°) is again partly lost when the corpuscles are placed at a higher temperature (above 25°).

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